GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR AND NEURTURIN IN THE REGULATION OF SPERMATOGENESIS

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals, and on some unpublished results.

I. Meng X, Lindahl M, Hyvönen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, Pichel JG, Westphal H, Saarma M, Sariola H. (2000) Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. **Science** *287, 1489-1493*. (Supplementary Material for publication I. http://www.sciencemag.org/feature/data/1046816.shl, Web Figure 1-5).

II. Creemers LB, Meng X, den Ouden K, van Pelt AMM, Izadyar F, Santoro M, Sariola H, de Rooij DG. (2001) Transplantation of germ cells from GDNF overexpressing mice to host testes depleted from endogenous spermatogenesis by fractionated irradiation. **Biology of Reproduction**, *under revision after review*.

III. Meng X, de Rooij DG, Westerdahl K, Saarma M, Sariola H. (2001) Promotion of seminomatous tumors by targeted overexpression of GDNF in mouse testis. **Cancer Research** *61, 3267-3271*.

IV. Meng X, Pata I, Pedrono E, Popsueva A, de Rooij DG, Jänne M, Rauvala H, Sariola H. (2001) Transient disruption of spermatogenesis by deregulated expression of neurturin in testis. **Molecular and Cellular Endocrinology** *183, 33-39*.

ABBREVIATIONS

Aal	A aligned spermatogonia
Apr	A paired spermatogonia
As	A single spermatogonia
ARTN	Artemin
cDNA	complementary DNA
CIS	carcinoma *in situ*
CS	classic seminoma
DNA	deoxyribonucleic acid
EC	embryonal carcinoma
EDCs	endocrine disrupting chemicals
EGF	epidermal growth factor
FMTC	familial medullary thyroid carcinoma
FSH	follicle-stimulating hormone
GCT	germ cell tumour
GDNF	glial cell line-derived neurotrophic factor
GFRα	GDNF family receptor alpha
GnRH	gonadotropin-releasing hormone
GPI	glycosylphosphatidyl inositol
HSCR	Hirschsprung's disease
LH	luteinizing hormone
kb	kilobase pair
kDa	kilodalton
MAPK	mitogen-activated protein kinase
MEN2	multiple endocrine neoplasia type 2
MTC	medullary thyroid carcinoma
mRNA	messenger ribonucleic acid
NRTN	neurturin
NSE	nonseminoma
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PGC	primordial germ cell
PI3-K	phosphatidylinositol 3-kinase
PLAP	placental alkaline phosphotase
PSPN	persephin
PTC	papillary thyroid carcinoma
RET	**re**arranged during **t**ransfection
SCF	stem cell factor
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SE	seminoma
SS	spermatocytic seminoma
TGCT	testicular germ cell tumour
TGFβ	transforming growth factor beta

ABSTRACT

Spermatogenesis is a complex process requiring not only hormonal regulation but also a large number of well-orchestrated paracrine and autocrine mechanisms that control cell proliferation and differentiation. This thesis presents my work on glial cell line-derived neurotrophic factor (GDNF) and neurturin (NRTN) in spermatogenesis and the pathogenesis of germ line tumours.

GDNF is a multifunctional molecule during embryogenesis and in some adult tissues. In the testis, Sertoli cells express GDNF while spermatogonial cells, the undifferentiated germ cells, express the GDNF receptors, Ret and GDNF family receptor α1 (GFRα1). A factor homologous to GDNF, NRTN, is also expressed by Sertoli cells, while its ligand binding coreceptor GFRα2 is expressed prominently by spermatids and weakly by spermatocytes. These expression patterns suggest that GDNF and NRTN could be paracrine regulators of germ cell development.

To approach the functions of GDNF and NRTN in spermatogenesis I used transgenic mouse models. Due to the early lethality of GDNF-deficient mice in the first postnatal day, spermatogenesis was analysed in heterozygous (GDNF+/-) mice. The transgenic mice with testis-specific overexpression of GDNF and NTRN were generated under the human elongation factor 1α (EF-1α) promoter. The dosage of GDNF regulates cell fate decision of undifferentiated spermatogonia, as the low dosage in GDNF+/- mice leads to the depletion of spermatogenic cells, whereas overexpression of GDNF promotes self-renewal of spermatogonial stem cells. By transplanting GDNF-expressing cells to irradiated wild type testes, the arrest of differentiation is shown to be cell-autonomous. GDNF-expressing spermatogonia do not differentiate in the new host, but are capable of repopulating the seminiferous tubules. Testis tumours frequently develop in transgenic mice with testes-specific overexpression of GDNF. We found high activity of AKT (protein kinase B) in the downstream pathway of GDNF/Ret signalling that may be essential for the formation of testicular tumours. Characteristics of these tumours represent the first transgenic model for human seminoma. Our data from the testis-specific overexpression of NRTN and GDNF suggest that NRTN and GDNF have different functions. NRTN may have a role in the regulating of the maintenance and development of spermatocytes and spermatids, and the fluid production or reabsorption of seminiferous tubules.

This GDNF-overexpressing transgenic mouse line serves as a powerful tool to study the pathogenesis of seminoma and to test novel therapies and diagnostic methods. Excessive stem cell self-renewal in the presence of a high GDNF dosage suggests that GDNF may also be useful *in vitro* for multiplying the stem cell population prior to germ cell transplantation, which might be beneficial for the patients who need to cryopreserve spermatogonic cells prior to oncogenic therapy.

***Key words*:** GDNF, NRTN, Ret, GFRα, AKT, testis, spermatogenesis, seminoma, transplantation.

1. REVIEW OF THE LITERATURE

1.1 Testicular germ cell development

1.1.1 Spermatogenic cells of the foetal and prepuberal mouse

Primordial germ cells (PGCs), the common germ cell lineage in both male and female embryos, are derived from a small population of the epiblast adjacent to the extra-embryonic ectoderm cells in mouse at embryonic day 6.0 (E6.0) (Lawson and Hage 1994). PGCs are recognisable owing to their staining for alkaline phosphatase at E7.25 (Ginzburg et al. 1990). Subsequently, proliferating PGCs migrate from the base of the allantois along the hindgut to the genital ridges at E10.5-11.5. There they must make a big decision: to be in mitosis or meiosis, to become sperm or egg? The PGCs differentiate into precursor cells of either male or female gametes under the control of cell-cell interactions in the developing gonad (Gilbert 2000). The first stage in testis development is the initiation of testis cord formation at approximately E12.0 (Tilmann et al. 1999), when PGCs commit to become the precursors of male gametes. Then the PGCs become enclosed by supporting somatic cells, the precursor Sertoli cells. When the PGCs are enclosed in seminiferous cords they change morphologically and are called gonocytes, sometimes prospermatogonia, which are centrally located within the seminiferous cords. The gonocytes proliferate for a few days and then arrest in the $G_0/_1$ phase of the cell cycle until soon after birth (Peters 1970). The gonocytes resume proliferation and migrate to establish contacts with the basement membrane of seminiferous tubules within a few days after birth and to give rise to adult type spermatogonia (Orth et al. 1997).

In postnatal day 6, the seminiferous epithelia contain only primitive type A spermatogonia and Sertoli cells. Type A and type B spermatogonia are present by day 8. At day 10, meiotic prophase is initiated. The germ cells reach the early and late pachytene stages by days 14 and 18, respectively. Secondary spermatocytes and haploid spermatids appear in increasing numbers between days 18 and 20 (Bellvé et al. 1977), thereby signifying the onset of spermatogenesis. The seminiferous cord has acquired a lumen in many areas by day 16, when the Sertoli tight junction (blood-testis barrier) is formed (Bellvé et al. 1977). The occluding Sertoli cell junctions are the major structural components of the blood-testis barrier (Gilula et al. 1976; Byers et al. 1991). The appearance of a lumen in many areas of the seminiferous cords at this time indicates the impermeability of the Sertoli junctions. With maturation of the tight junctions, the seminiferous epithelium is effectively divided into two compartments. The basal compartment in which the spermatogonia and early primary spermatocytes reside, and the adluminal compartment in which the more advanced spermatocytes and spermatids are secluded (Dym 1973; Byers et al. 1991; Pelletier and Byers 1992). This barrier creates a unique microenvironment in which germ cells except spermatogonia and early spermatocytes are segregated from the systemic circulation.

1.1.2 The adult testis

In the seminiferous tubules the Sertoli cells support the developing germ cells. The tight junctions between the Sertoli cells provide a blood-testis barrier, resulting in tubular lumen and fluid having quite a different composition from plasma (Bellvé et al. 1977; Gilula et al. 1976; Byers et al. 1991). The interstitial space between the capillary endothelium and tight junctions of seminiferous tubule contains substances and nutrients for Leydig cells. Leydig cells produce the major male sex hormone, testosterone. Peritubular myoid cells surround the seminiferous tubule and are in contact with the basal surface of Sertoli cells. Cell-cell interactions between all three somatic cell types are possible (Fig. 1; Parvinen et al. 1982; Skinner 1991; Gnessi et al. 1997). However, Sertoli cells appear to be the primary somatic cell type to directly interact with the developing germ cells.

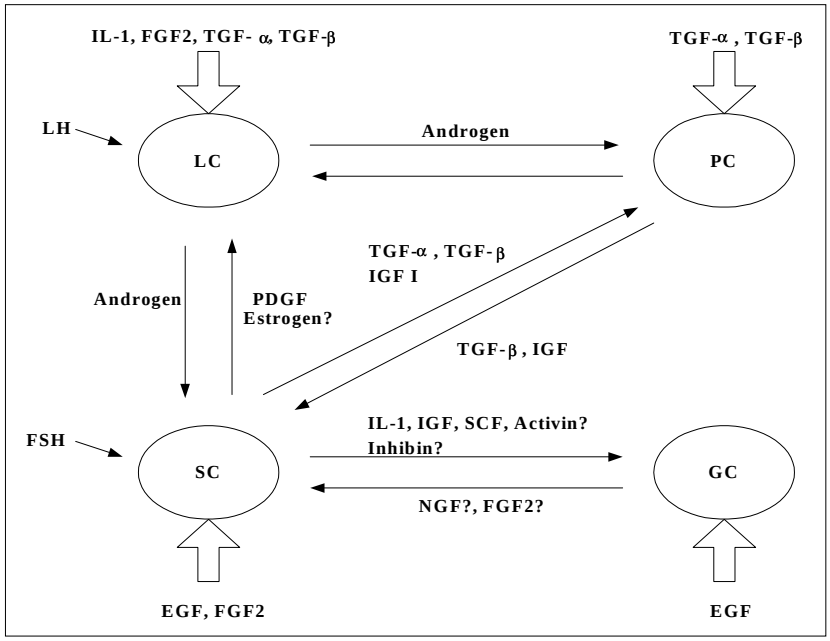

Figure 1. Cell types and some examples of their interactions in adult testis. Cell types: GC, germ cells; LC, Leydig cell; PC, peritubular cell; SC, Sertoli cell. Potential local factors: EGF, epidermal growth factor; FGF2, fibroblast growth factor2; FSH, follicle-stimulating hormone; IGF, insulin-like growth factor; IL, interleukin; LH, luteinizing hormone; NGF, nerve growth factor; PDGF, platelet-derived growth factor; SCF, stem cell factor; TGF, transforming growth factor.

1.1.3 Spermatogenesis

In mammals spermatogenesis occurs within seminiferous tubules that release spermatozoa into the rete testes, which connect the seminiferous tubules to the epididymis. The process in which spermatogonia form spermatozoa is called spermatogenesis. This complex process involves a series of mitotic divisions of spermatogonial cells, two meiotic divisions of spermatocytes, extensive morphological remodelling of the spermatids during spermiogenesis, and the release of the maturing spermatozoa into lumen by spermiation. In the mouse, the entire developmental process from stem cell to spermatozoon takes 34.5 days. The spermatogonial stage lasts 8 days, meiosis lasts 13 days, and spermiogenesis takes up another 13.5 days (Clermont 1972). The spermatogonial stem cells are capable of self-renewal and commitment of differentiation and, therefore, spermatogenesis continues via a cyclic process during the life of the male. The precise combinations of various generations of spermatocytes and spermatids derived from individual spermatogonia reflect waves of spermatogenesis that are not random but instead occur in a specific cyclic manner referred to as stages (Russell et al. 1990). The succession of these stages of germ cells is referred to as a cycle of the seminiferous epithelium (Russell 1990).

1.1.3.1 Mitotic phase of spermatogenesis

A. Stem cells and undifferentiated spermatogonia

During the proliferative phase, spermatogonial stem cells in the basal compartment of seminiferous tubules undergo mitotic divisions giving rise to both new stem cells and committed precursors that continue to divide and differentiate into spermatocytes. In mammals spermatogonial stem cells are A single (As) spermatogonia that either renew themselves or produce A paired (Apr) spermatogonia destined to differentiate (Huckins 1971a,b; Oakberg 1971; Lok et al. 1983; de Rooij 2001). In turn, the Apr divides into chains of A aligned (Aal) spermatogonia that undergo mitotic divisions but remain as a syncitium through the intercellular cytoplasmic bridges (Fig. 2) (reviewed by de Rooij 2001). As, Apr and Aal compose the undifferentiated spermatogonia.

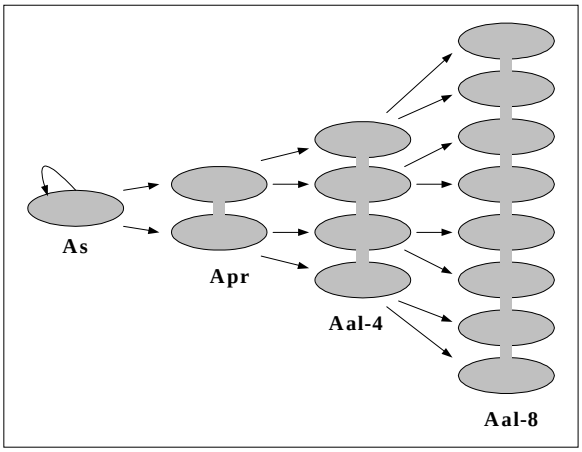

Figure 2. The divisions and differentiation of undifferentiated spermatogonial cells. Note: As, A single spermatogonia; Apr, A paired spermatogonia; Aal, A aligned spermatogonial cells; numbers 4 and 8 refer to the cell number of the interconnected spermatogonial chain.

Two possible mechanisms for spermatogonial stem cell renewal and commitment towards differentiation have been presented (Fig. 3) (Reviewed by de Rooij 2001). The commonly favoured symmetric division theory implies that a stem cell gives rise to either two stem cells or two Apr spermatogonia. The asymmetric division theory suggests that the stem cell, through an asymmetric division, produces a new stem cell and another "As" that is destined to Apr differentiation and, therefore, this "As" cell is no longer a true stem cell. Only little evidence for the possibility of asymmetric division has been presented. In rat, some As spermatogonia retain incorporated 3-(H) thymidine for a very long time, indicating that these cells have a very long cycle while other As spermatogonia lose their label more quickly. It was proposed that the long-cycling cells are true stem cells and the short-cycling cells are destined to become Apr spermatogonia (Huchins 1971b).

How to keep a stem cell as a stem cell? Very little is known about the answer. After severe cell damage, stem cell renewal is favoured over differentiation and the period of at least As, Apr, and Aal spermatogonia is extended (Van Beek et al. 1990; de Rooij 2001). After exposure to irradiation and toxic substances, spermatogonia and some of the stem cells undergo degeneration leading to germ cell depletion. The surviving spermatogonial stem cells repopulate the seminiferous epithelia, and initially stem cells renewal overrides differentiation. During the first six divisions after irradiation stem cells virtually only self-renew (Van Beek et al. 1990). This finding suggests that there must be a regulatory mechanism controlling the cell fate decision of stem cells. In normal seminiferous epithelia, the ratio between self-renewal and differentiation of spermatogonia should be about 1:1 (Reviewed by de Rooij 2001). More self-renewal than differentiation of stem cells would reduce the seminiferous epithelia and may raise the risk of germ cell tumours.

If differentiation overrides, the stem cell pool is consumed and may vanish, resulting in Sertoli cell-only seminiferous tubules.

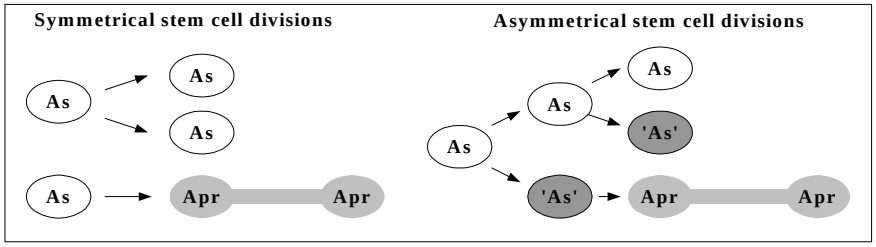

Figure 3. Two hypothetical models for stem cell self-renewal and differentiation. Modified according to de Rooij 2001.

B. Differentiating spermatogonia

The last generation of undifferentiated spermatogonia, Aal spermatogonia do not divide but differentiate to A1 spermatogonia. Few Apr, and even fewer As spermatogonia can also differentiate to A1 (reviewed by de Rooij 2000). C-kit, Dazl RNA protein, cyclin D2, and retinoic acid have been implicated in this differentiation step (reviewed by de Rooij 2001). Spermatogonia further undergo six mitotic divisions resulting in generations of more differentiated spermatogonia: A2 to A4 spermatogonia, intermediate spermatogonia, and type B spermatogonia that divide and give rise to prophase spermatocyte proleptotene (Fig. 4). A1, A2, A3, A4, In, and B spermatogonia are grouped to differentiating spermatogonia (reviewed by de Rooij and Russell 2000; de Rooij 2001).

The lack of fine regulation of the density of spermatogonial stem cells results in an uneven density of A1 spermatogonia distribution in different segments of seminiferous tubules. An equal density of spermatocytes is obtained by apoptosis of A2, A3 or A4 spermatogonia to remove the surplus cells. Germ cell apoptosis plays an important role in limiting the density of germ cells thus guaranteeing their optimal development, and Sertoli cells have been shown actively controlling the number of germ cells (de Rooij and Loc 1987; Lee et al. 1997).

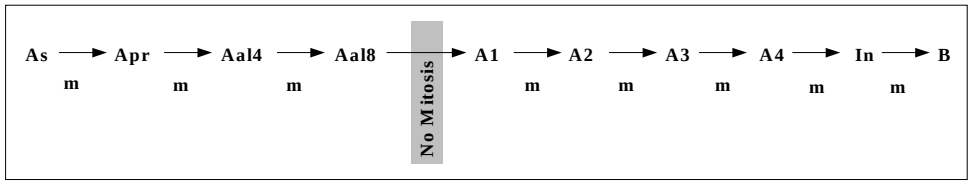

Figure 4. Progression of spermatogonial divisions. According to de Rooij and Russell, 2000. m: mitosis.

C. Spermatogonial transplantation

The proliferatively active spermatogonial stem cells offer a potential to restore spermatogenesis by stem cell transplantation. Germ cell transplantation is an important tool in restoring fertility, for generating a genetically modified male germ line, and also as a research tool for germ cell biology. The pioneer work in the development of the technique using microinjection to introduce the donor cells into seminiferous tubules was performed by Brinster and Zimmermann (1994) and Brinster and Avarbock (1994). As an alternative to intratubular infusion of germ cells by microinjection, injections into efferent ducts as well as into the rete testis of the host mice have been successfully employed (Ogawa et al. 1997).

The ability to cryopreserve donor germ cells for a long period of time prior to the transplantation has been established (Avarbock et al. 1996; Nagano and Brinster 1998). The transplantation of spermatogonial stem cells cultured for four months has also been successful (Nagano et al. 1998). Therefore, these techniques provide possibilities for multiplication and manipulation of male germ line cells, and also provide the clinical possibility to reintroduce the preserved germ cells after cancer therapies into the male gonad that has been damaged by the therapy.

Electron microscopy of the host testes has shown focal arrangements of quantitatively and qualitatively normal donor spermatogenesis in the transplanted testis (Russell et al. 1996). A time course study after transplantation showed that most transplanted cells degenerate and disappear from the testis during the first few weeks (Parreira et al. 1998). The first meiotic donor germ cells appear one month after transplantation and after two months only 1% of all seminiferous tubules contain meiotic germ cells. The xenogenetic transfer of germ cells between species has shown that the donor spermatogonia are able to repopulate the basal compartment of the host seminiferous tubules. Some of the host species can support normal or abnormal donor spermatogenesis, some do not allow the xenogenetic donor germ cell differentiation at all (reviewed by Ogawa 2001).

There are several important new findings arising from experiments using germ cell transplantation. First, it became obvious that the blood-testis barrier of the adult testis does not hinder the migration of transplanted spermatogonia cells from the adluminal compartment to the basal compartment of the seminiferous epithelium. Second, the appearance of sperm in w/w mutant mice arising from the donor spermatogonia revealed that germ cells without functional c-kit receptors are not able to enter the process of spermatogenesis (Brinster and Zimmermann 1994).

1.1.3.2 Meiotic phase of spermatogenesis

The last mitotic division of spermatogonia (B spermatogonia) gives rise to the young spermatocyte, the preleptotene spermatocyte. During the prophase, the nuclei of cells increase in size. The preleptotene spermatocyte progresses into leptotene, zygotene, pachytene, and diplotene primary spermatocytes that undergo the meiotic DNA replication and recombination of homologous chromosomes and two meiotic divisions.

In the preleptene-leptotene transition, the cells move from basal compartment towards the adluminal compartment of the blood-testis barrier. In the leptotene phase, the homologous chromosomes become condensed but remain unpaired. The meiotic recombination begins before chromosome synapsis at the sites of double strand breaks form at early meiotic prophase (Hawley and Arbel 1993). In the zygotene phase, the synapsis of homologous chromosome occurs and produces the structure called the synaptonemal complex, which is fully formed by the late pachytene phase. In pachytene cells, the chromosomes have become fully paired and remain so for a long time (in mammals typically 1.5-2 weeks). Genetic recombination known as crossing over also occurs during this period. The diplotene phase is rather brief. In the diplotene phase, the synaptonemal complex dissipates and chromosomes separate from each other except at the regions of chiasmata. The metaphase, anaphase and telephase are referred to as the first meiotic division, or meiosis I. The cells now become secondary spermatocytes which rapidly undergo the second meiotic division or meiosis II resulting in haploid spermatids (Russell et al. 1990).

1.1.3.3 Spermiogenic phase of spermatogenesis

During the third or spermiogenic phase, the early round spermatids transform without cell division into mature spermatozoa. This phase takes almost two weeks in mouse and can be divided into 16 steps according the morphological characteristics of the formation of the flagellum, acrosome, elongation and the condensation of the nucleus, as well as nuclear DNA packing (Russell et al. 1990). The elimination of the cytoplasm together with other changes results in the reduction of the volume of spermatids to approximately 25% of their original size.

The final maturation and capacitation of sperm take place in the epididymal ducts, which develops from the cranial mesonephros during embryogenesis. Growth factors and their receptors regulate the epithelial differentiation of the epididymal duct. Loss-of-function of bone morphogenetic protein-8a (Bmp-8a) leads to the degeneration of the epididymal epithelium (Zhao et al. 1998). Overexpression of vascular endothelial growth factor (VEGF) causes the dilation of the ductus epididymis with regional epithelial hyperplasia (Korpelainen et al. 1998). The inactivation of the c-Ros orphan receptor tyrosine kinase results in an abnormal epithelial differentiation in the cranial part of epididymis, and therefore causes male infertility (Sonnenberg-Riethmacher et al. 1996). The spermatids produced are conducted from the seminiferous tubules to the epididymis by the fluid floating through the tubular lumen, rete testis, and efferent ducts to the epididymis. The effect of the inefficient fluid reabsorption or excessive production may result in infertility. The estrogen receptor knockout (ERKO) mice are infertile due to the problem of fluid reabsorption in the efferent ductules, and to a lesser extent, in the initial segment of the epididymis (Eddy et al. 1996; Hess et al. 1997).

1.1.4 The impact of Sertoli cells

The Sertoli cell, the only somatic cell type within the seminiferous tubule, plays numerous essential roles in the regulation of spermatogenesis (Parvinen et al. 1982, 1986; reviewed by Russell and Griswold 1993). These range from the association of Sertoli

cells with germ cells and their structural role in the establishment of the blood-testis barrier to the importance of the paracrine regulation of germ cell differentiation (reviewed by Bardin et al. 1988; Skinner 1991; Gnessi et al. 1997). An individual Sertoli cell can be in contact with five adjacent Sertoli cells at the basal surface and 47 adjacent germ cells at various stages of development (Russell et al. 1983). The ability of an individual cell to be in contact with more than 50 adjacent cells indicates the importance of interactions between these cells. The Sertoli cell receives nutrition and signals from the circulatory system and surrounding cells, and then transports nutrients to germ cells and adjusts the input signals to mediate germ cell development by both paracrine products and modulating its own function. In addition, the Sertoli cell also has the functions of phagocytosis and pinocytosis to remove the dead cells (reviewed by Russell and Griswold 1993).

1.1.5 Germ cell apoptosis during spermatogenesis

Extensive cell death is a striking feature of spermatogenesis. Apoptosis occurs at a baseline level in adult testis, and 25 to 75% of the theoretically expected germ cell yield is lost during spermatogenesis (Oakberg 1956; Huckins 1978; de Rooij and loc 1987; Wang et al.1998a). In the postnatal murine testis, apoptosis is detected in spermatogonia and spermatocytes (Allan et al. 1987; Bartke 1995; Blanco-Rodriguez 1998; Embree and Boekelheide 2000). Apoptotic cell death plays an important role during development by regulating the size of the germ cell lineage in relation to its local environment, the survival itself being dependent upon the availability of local factors and their regulatory stimuli (de Rooij and Loc 1987; Lee et al. 1997; Conlon and Raff 1999). Sertoli cells actively limit the number of germ cells at least by producing death factors such as FasL, which signals germ cell death (Lee et al. 1997). It is also assumed that spermatogonia and spermatocytes with chromosomal abnormalities during replication and division might be eliminated through apoptosis by unknown mechanism(s) in order to keep the fidelity of the genome (Allan et al. 1987). In addition to the role of growth and survival factors in the development and maintenance of normal germ cell function, it has been increasingly evident that cell death is important for testicular homeostasis (Matsui 1998; Embree and Boekelheide 2000). Two separate transgenic mouse models overexpressing anti-apoptosis factors, either BclxL or Bcl2, in testis germ cells show severely abnormal spermatogenesis accompanied by sterility (Furuchi et al. 1996; Rodriguez et al. 1997). Moreover, spermatogenesis is impaired in null mice for the pro-apoptosis factor bax and they are characterised by atrophic testis and male infertility (Knudson et al. 1995).

The regulatory mechanisms of germ cell apoptosis are still not fully understood. However several factors including the SCF/c-kit system, Fas/fas ligand system, Bcl-2 family proteins, and bone morphogenetic protein-8a or -8b (Bmp-8a or -8b) are involved in the regulation of germ cell apoptosis. Apoptosis can be triggered by the absence of a trophic signal, which normally promotes cell survival. For instance, gene disruption of Bmp-8a or -8b shows increased germ cell apoptosis (Zhao et al. 1996, 1998). c-kit and its ligand stem cell factor (SCF) are crucial for the survival of germ cells. The injection of a monoclonal antibody to c-kit to inhibit SCF binding to the receptor leads to decreased numbers of differentiating spermatogonia (Yoshinaga et al. 1991) and high levels of apoptosis in germ cells undergoing mitosis and meiosis (Packer et al. 1995). There have

also been a number of studies showing that withdrawal of follicle stimulating hormone (FSH) and testosterone stimulation of the testis results in apoptosis (reviewed by Sinha and Swerdloft et al. 1999). SCF levels are increased by FSH (Yan et al. 1999) and this decreases the number of germ cell undergoing apoptosis (Yan et al. 2000a,b). *In vitro* data showed the involvement of Bcl-2 and Bcl-w in germ cell apoptosis (Yan et al. 2000c,d). Apoptosis is often a consequence of an arrest of germ cell differentiation (Nantel et al. 1996; Ashley 2000). Cell death can be induced by noxious agents or pathological conditions, for instance, ionising radiation, cytotoxic drugs, hyperthermia and ischaemia, and an over-load of tubular fluid (Allan et al. 1987; Eddy et al. 1996; Hess et al. 1997).

1.1.6 Hormonal control of spermatogenesis

Normal spermatogenesis requires a fully functional hypothalamo-hypophysial system (reviewed by Matsumoto 1989, 1991; Huhtaniemi and Toppari 1998). The two major pituitary gonadotropins, luteinizing hormone (LH) and FSH, are required for spermatogenesis (Finkel et al. 1985).

LH indirectly regulates spermatogenesis via stimulating the testosterone (T) production of Leydig cells that have the receptor for LH (Catt et al. 1980). The high local concentration of T produced by Leydig cells binds to intracellular receptors located in Sertoli cells and peritubular myoid cells to regulate spermatogenesis (Sar et al. 1993). Most recently, two different research groups simultaneously generated LH receptor deficient mice which show a similar phenotype to the human patients with an inactivating LHR mutation (Beck-Peccoz et al. 2000; Zhang et al. 2001; Lei et al. 2001). Spermatogenesis in these mutant mice is arrested at the round spermatid stage and Leydig cell numbers are dramatically reduced (Zhang et al. 2001; Lei et al. 2001).

The importance of FSH for the initiation of spermatogenesis has been challenged by several recent findings. First, in men with inactivating mutations of the FSH receptor gene, spermatogenesis proceeds to completion though sperm counts are low and testis size is reduced (Tapanainen et al. 1997). Second, spermatogenesis is complete in the FSH β-subunit deficient mice that were fertile. The testes in these mice are smaller with a decreased Sertoli cell number and low germ cell count (Kumar et al. 1997). Third, in the hypogonadotropic (hpg) mutant mouse that has undetectable levels of both LH and FSH, due to lack of gonadotropin-releasing hormone (GnRH), spermatogenesis can be initiated by T but it has a low sperm output (Singh et al. 1995). Despite these data, the concept that FSH plays critical roles in establishing normal spermatogenesis is reinforced by the following data. Many patients with Kallman's syndrome cannot achieve normal spermatogenesis without using a combination of FSH and LH (reviewed by de Kretser et al. 2000).

FSH binds to a specific G protein-coupled membrane receptor on Sertoli cells and controls spermatogenesis through regulation of Sertoli cell function (Griswold 1993). FSH plays a key role in controlling overall sperm production by stimulating Sertoli cell proliferation during the prenatal and postnatal period and by regulating the secretion of a

number of Sertoli cell products, *e.g*. androgen binding protein (ABP), transferrin, testibumin, inhibin, transforming growth factor-β (TGF-β), Müllerian inhibiting substance (MIS), insulin-like growth factor (IGF) binding protein 3, SCF, polyamines, plasminogen activators, lactate, and pyruvate during different periods of development (reviewed by Russell and Griswold 1993). The link between the classical hormonal regulation of spermatogenesis and SCF has been strengthened by the observation that SCF levels are increased by FSH (Yan et al. 1999). This stimulation is translated into increased numbers of germ cells (Yan et al. 2000a). These results provide an excellent example of how Sertoli cells transmit the hormonal regulation of spermatogenesis to germ cells. FSH may be a prerequisite of T action during development. Assuming that the effects of FSH and T are mediated by Sertoli cell, it has been suggested that the Sertoli cell is particularly vulnerable at certain times to the loss of hormonal stimulation. Meachem and co-workers (1998) showed that the neutralisation of FSH *in vivo*, using a polyclonal antibody against FSH, could impair the action of T in restoring spermatogenesis in the testosterone-estradiol suppressed or GnRH-immunised models.

Although the importance of T in the maintenance of spermatogenesis is not disputed, evidence has been adduced from the outcome of mutations in the androgen receptor (AR) which can cause total or partial androgen insensitivity leading to a range of patterns of abnormal sexual differentiation (Quigley et al. 1995; Tut et al. 1997; Wang et al. 1998b; Dowsing et al. 1999). The hgp-mouse model of GnRH-deficiency proposed that the most T-sensitive step is the conversion of spermatocytes to spermatids (Singh et al. 1995). Both T and FSH stimulate the production of the cell adhesion molecule, N-cadherin, which may be involved in the binding of spermatids to Sertoli cells (Perryman et al. 1996). On the basis of their *in vivo* studies, O'Donnell et al (1996) proposed that T is essential in preventing the premature desquamation of round spermatids from the seminiferous epithelium. Further evidence for the important role played by T in spermatogenesis was demonstrated by the administration of T to normal men leading to suppression of sperm output: T suppressed FSH and LH, which later lead to a decrease in intra-testicular T levels (Matsumoto et al. 1983; Zhengwei et al. 1998).

1.1.7 Cell-cell interactions in the regulation of spermatogenesis

The identification of multiple testicular cell types has initiated studies of cell-cell interactions that have continued for the past 100 years. The research in the area has recently been increasing. In 1865, Enrico Sertoli described special branched cells in the seminiferous tubules of the human testis. Sertoli postulated "the function of the branched cells is linked to the formation of spermatozoa" (reviewed by Skinner 1991). Further analysis of the Sertoli cell and its association with the developing germ cells lead to the concept that this "nurse cell" is essential for the germ cell and the process of spermatogenesis via cell-cell interactions (Parvinen et al. 1982, 1986; reviewed by Skinner 1991; Jégou and Shap 1993; Russell and Griswold 1993; Gnessi et al. 1997; Mruk and Cheng 2000). The cell-cell interactions include an environmental interaction, nutritional interaction and regulatory interaction (reviewed by Skinner 1991). The lack of physical contact between Leydig cells and Sertoli cells or germ cells indicates that the nutritional and environmental interactions between these cells are not likely. However, all

three types of interactions are possible between Sertoli cells and germ cells and are crucial in the direct regulation of spermatogenesis.

Several aspects of testis cell biology that are integrally associated with local cellular interactions involved proliferation, growth, differentiation, and germ cell movement by regulating the turnover of tight junctions, anchoring junctions, and gap junctions during the process of germ cell movement throughout spermatogenesis (Mruk and Cheng 2000). The cytoarchitectural arrangements between Sertoli cells and the developing germ cells provide one of the most sophisticated environmental cell-cell interactions. The Sertoli cell is required to maintain the germ cell syncitium that is connected to all cells derived from an initial clone of cells. The presence of junctional contacts between Sertoli cells and germ cells suggests that these environmental interactions may be needed for cell attachment and association. The continuous nature of germ cell development and the presence of the cycle of the seminiferous epithelia indicate that environmental interactions between Sertoli cells and germ cells are dynamic and require a rapid rate of tissue remodelling. The production of plasminogen activator and other protease activities of Sertoli cells may be needed in several aspects of tissue remodelling associated with translocation of spermatocytes, degradation of junctional complexes, and release of mature spermatozoa into lumen of tubule. The function and control of the proteolytic activities in remodelling might be also regulated by paracrine local factors secreted by Sertoli cells (Mruk and Cheng 2000).

The nutritional interaction between Sertoli cells and germ cells is required due to the presence of the blood-testis barrier (Gilula et al. 1976; Bellvé et al. 1977; Christensen et al. 1985; Byers et al. 1991). One of the major functions for the Sertoli cell is to transport essential components to the spermatogenic cells sequestered in a unique serum-free microenvironment. In the adult testis an interaction, a desmosome-gap junction, forms between Sertoli cells and germ cells. These gap junctions are mainly required for transporting compounds between Sertoli cells and germ cells (Russell et al.1983).

Regulatory interactions occur among all testicular cell types. It has been suggested that the local production and action of growth factors are required to regulate male gonadal function (reviewed by Bellvé and Zhang 1989). Further investigation of potential regulatory interactions between Sertoli cells and germ cells requires the elucidation of the paracrine factors involved (Pescovitz et al. 1994). Regulatory interactions between Sertoli cells and germ cells were proposed when the concept of paracrine factors and local trophic factors was developed. Recent studies have identified numerous secretory products for specific testicular cell types. These products range from steroids to secreted proteins. Interestingly, most of the secretory products identified appear to be involved directly or indirectly in cell-cell interactions. These products include transport/binding proteins, proteases, extracellular matrix components, growth factors, and cellular metabolites.

1.1.8 Autocrine and paracrine modulators of testicular function

A rapidly increasing number of testicular factors are now being identified that modulate spermatogenesis in a paracrine or autocrine fashion (Table 1). The physiological

functions of many of the factors remain to be identified. The Sertoli cell is the major secretory cell type in the seminiferous epithelium (reviewed by Bardin et al. 1988; Jégou and Shap 1993; Russell and Griswold 1993; Gnessi et al. 1997). As such, Sertoli cells interact with germ, Leydig, and peritubular myiod cells biochemically via paracrine factors produced by Sertoli cells in addition to the physical contacts between these cells. Moreover, paracrine factors released by germ cells or peritubular myoid cells can also regulate Sertoli cell and testicular functions illustrating the existence of a two-way trafficking between these cells (reviewed by Jégou and Shap 1993; Russell and Griswold 1993; Spiteri and Nieschlag 1993; Pescovitz et al. 1994; Huhtaniemi and Toppari 1998). Spermatogenesis defects in men are not improved by gonadotropin, which raises the possibility that abnormalities of local paracrine or autocrine factors might be underlying the defects. It has been estimated that the abnormalities of the possible local factors may contribute to 40-80% of infertility cases in men with oligozoospermia or azoospermia (reviewed by Baker et al. 1986; Matsumoto 1991, Escalier 1999). There is accumulating evidence that growth factors may function in an autocrine or paracrine fashion to stimulate mitosis, growth, angiogenesis, and differentiation in the testis (reviewed by Ackland et al. 1992). *In vivo*, both gene disruption and transgenic mice have showed the importance of paracrine or autocrine regulation of growth factors and receptors in spermatogenesis. For instance, spermatogenesis abnormalities have been found in BMP-8a and -8b deficient mice (Zhao et al. 1996, 1998). SCF is expressed by Sertoli cells, the c-kit receptor is expressed in germ cell. Mice harbouring mutations at the dominant white spotting (*W*) (Russell 1949) or steel (*Sl)* (Sarvella and Russell 1956; Reith and Bernstein 1991) loci exhibit similar pleiotropic abnormalities. Male mice with natural mutations in either *W* or *Sl* genes encoding for c-kit/SCF are sterile due to the lack of germ cells in the testis. Thus, the SCF/c-kit interaction is essential for normal germ cell development (reviewed by Russell 1979; Dubreuil et al. 1990; Vincent et al. 1998; Yan et al. 1999; 2000a,b). Both reduction of circulating epidermal growth factor (EGF) by sialoadenectomy and EGF overexpression result in hypospermatogenesis and male sterility (Tsutsumi et al. 1986; Liu et al. 1994; Wong et al. 2000). Proper EGF expression is thus important for the completion of spermatogenesis.

Table 1. Presumed functions of some paracine/autocrine factors in the testis

Factor	Site	Function or presumed function
ABP	SC	Androgen transport and storage
Transferrin	SC	Iron transport
Inhibin	SC, LC	Inhibits FSH secretion; Inhibitory autocrine factor. Stimulates LC steroidogenesis.
Activin	SC, LC	Stimulates Sg; Stimulatory autocrine factor. Inhibits LC steroidogenesis.
IGF-I	LC, SC	Stimulates LC steroidogenesis, peritubular cell, Sg, early Sd; Stimulatory factor.
FGF-2	LC, SC, GC,PC	Stimulates LC steroidogenesis, pachytene Sp.
TGFα	SC	Stimulates PC, LC, GC growth.
TGFβ	SC, LC, PC	Modulates LC steroidogenesis, inhibits PC growth.
MIS	SC	Stimulates prepubertal GC, Sg, and Sp growth.
IL-1	SC, LC	Stimulates PGC, Sg, and Sp growth.

IL-6	SC	Stimulates GC growth.
SCF	SC	Stimulates PGC development and Sg proliferation and survival.
α-MSH	LC	Stimulates SC proliferation and function.
β-Endorphin	LC	Inhibits SC proliferation and function.
NGF	GC	Regulates SC ABP production & other function?
TNF-α	GC	Modulates SCF function.
Polyamines	SC	Regulates GC proliferation and differentiation. Inhibits FSH binding to receptor.
Estradiol	SC	Prepubertal stimulatory autocrine factor. Inhibits LC steroidogenesis.
EGF	GC, SC	GC survival, regulates T production of LC, affects SC.
BMP-8a, -8b	GC	GC survival.
PDGF	SC	Stimulates adult Leydig cell development

ABP, androgen binding protein; FGF-2, fibroblast growth factor-2; GC, germ cell; LC, Leydig cell; IGF, insulin-like growth factor; IL, interleukin; MIS, anti-Müllerian hormone; MSH, melanocyte-stimulating hormone; NGF, nerve growth factor; PC, peritubular cell; SC, Sertoli cell; Sg, spermatogonia; Sp, spermatocytes; TGF, transforming growth factor; TNF, tumour necrosis factor; EGF, epidermal growth factor; BMP-8a and -8b, bone morphogenetic protein-8a and -8b; PDGF, platelet-derived growth factor.

1.2 Testicular germ cell tumours

1.2.1 Classification of testicular germ cell tumours

Male germ cell tumours (GCTs) comprise about 2% of all human malignancies. Over the past several decades, the incidence of GCTs has been steadily increasing in the Western world (Toppari et al. 1996; Bergström et al. 1996; Skakkebaek et al. 1987; Skakkebaek et al. 1998). Among testicular tumours, GCTs are the most common malignancy in young men at the age about 18-50 years (Skakkebaek et al. 1987; Bosl and Motzer 1997). Testicular cancer patients are treated with orchiectomy, and the treatment is thereafter dependent on histology and stage (Sundström et al. 2001). The majority of testicular GCTs (TGCTs) show exquisite sensitivity to cisplatinum-based treatment and irradiation. The prognosis of testicular cancer has improved considerably during the past 20 years owing to the development of cisplatin-based combination chemotherapy, however the mortality rate is still about 10% (Sundström et al. 2001). TGCT can be grouped clinically and histologically into two entities, seminoma (SE) (about half the TGCTs), which is subgrouped into classic seminoma (CS) and spermatocytic seminoma (SS), and nonseminomatous (NSE) GCT (≈40% of the cases). The remaining cases of GCTs comprise combined SE- and NSE-GCT components (Skakkebaek et al. 1987; Mostfi et al. 1987). NSEs exhibit various patterns of embryonal-like differentiation: primitive zygotic embryonal carcinoma (EC), embryonal-like somatically differentiated (teratoma), and extra-embryonally differentiated (choriocarcinoma, yolk sac tumour) phenotypes.

1.2.2 Carcinoma *in situ*

The histogenesis of GCTs in humans has been thoroughly investigated since 1972, when Skakkebaek reported on atypical spermatogonia in testicular biopsies of two infertile patients who later developed testicular cancer. These precancerous germ cells are known as carcinoma*insitu* (CIS) (Skakkebaek 1972; Skakkebaek and Berthelsen 1978). Morphologically, CIS consists of atypical, intratubular germ cells that are usually located in a single row at the basement membrane of seminiferous tubules. CIS cells are larger in diameter than normal spermatogonia; the nucleus is also larger than that of normal spermatogonia and usually contains several large nucleoli (Skakkebaek and Berthelsen 1978; Skakkebaek et al. 1987). The diameter of CIS tubules is usually decreased and the basement membrane is thickened. In most cases, the tubules with CIS do not have ongoing spermatogenesis and contain Sertoli cells as the only additional cell type (reviewed by Rørth et al. 2000). CIS cells probably originate from PGCs early during embryogenesis as a consequence of endocrinological imbalances. An excess of estrogens during early embryonic life might stimulate PGCs to acquire the tumorigenic potential of CIS cells (Skakkebaek et al. 1987, 1998; Dieckmann and Skakkebaek 1999). Ultrastructural studies have indicated a close resemblance of CIS cells to embryonal germ cells. Furthermore, the CIS cells have histochemical features in common with gonocytes (Skakkebaek et al. 1987), both cell types being rich in glycogen and placental alkaline phosphatase (PLAP). CIS cells express embryonic antigens (Jørgensen et al. 1993; Rajpert-De Meyts et al. 1996a,b). CIS cells have been found in individuals only a few months old, which also supports the assumption that these cells are of foetal origin. Aneuploid DNA contents of CIS cells were first observed in 1981 (Müller and Skakkebaek 1981). As the natural course of CIS is rather predictable, *i.e*. 50% of cases with CIS progress to invasive GCT within five years and in the long-term most, if not all, become invasive (Skakkebaek and Berthelsen 1978). Treatment is required to prevent the malignant transformation. There are four options for therapy: orchiectomy, cisplatin-based chemotherapy, low-dose radiotherapy, and temporal surveillance (Dieckmann and Skakkebaek 1999).

Probably, CIS remains in a dormant stage and divides at a very slow rate in the early years of childhood. Whether or not they give rise to GCT at this early point is unknown. After puberty, probably as a consequence of raised sex hormone levels, CIS cells start to divide. Several studies have shown that the CIS cells are the common precursor of adult and adolescent testicular GCTs for both SEs and NSEs (Skakkebaek and Berthelsen 1981; Skakkebaek et al. 1984; Skakkebaek and Berthelsen 1987; Dieckmann and Skakkebaek 1999). One exception is SS that appears to be a distinct entity that develops directly from germ cells (Skakkebaek et al. 1987; Dekker et al. 1992). Evidence for this assumption is provided by the lack of PLAP in the cells of this tumour type, the lack of CIS cells in the vicinity of these tumours, and different clinical behaviour. The infantile testicular tumours may also not be derived from CIS cells because they are not accompanied by CIS in the surrounding testicular tissue (Jørgensen et al. 1995). Almost all the GCTs which become manifest around the time of puberty and sexual maturations are NSE, whereas an increasing proportion of germ cell tumours are SEs with increasing age (Skakkebaek et al. 1998). There is more and more evidence that the CIS cell is a

malignant gonocyte with stem cell potential (Hanaoka et al. 1991; Matsui et al. 1992; Skakkebaek et al. 1998). This explains why an adult man can develop a NSE, which is a neoplastic recapitulation of embryonic growth. CIS lesions often develop into SE in one part, and NSE in another part of the same testicle. The capacity of CIS cells to develop into NSE tumours may decrease with age, as NSEs are rare after the age of 40 (Skakkebaek et al. 1998). In large patient materials no cases of CIS were found in men older than 50 years (Dieckmann and Loy 1996).

It is still unknown whether NSEs develop through a stage of SEs or progress directly from CIS to teratoma or embryonal carcinoma. It is unlikely that SE could transform into teratoma or embryonal carcinoma. This is suggested by the fact that the transplanted JKT-1 and JKT-HM SE cell lines in nude mice only formed SE-type tumours but not NSEs (Jo et al. 1999). Moreover, the differential expression of tumour markers might indicate that in the histogenesis of NSE there is not necessarily an intermediate stage of SE (Looijenga et al 1993; Rajpert-De Meyts et al. 1996b). Most likely, CIS may directly progress to either SE or to NSE, probably via intermediate stages of "SE-like CIS" or "NSE-like CIS" (Dieckmann and Skakkebaek 1999). Cytogenetic analyses have shown that CIS cells alongside SE have higher numbers of copies of chromosome 12 and 15 than CIS surrounding NSE (Looijenga et al. 1993). CIS cells as well as SE cells usually exhibit hypertriploid (triploid-tetraploid) DNA contents (De Jong et al. 1990). Immunohistologically, CIS associated with SE has a very high expression of PLAP and c-kit and a low expression of TRA-1-60, whereas CIS surrounding NSE has a high expression of TRA-1-60 and low or no expression of PLAP and c-kit (Rajpert-De Meyts et al. 1996a,b).

1.2.3 Seminomatous germ cell tumours

SE, approximately half of the cases of TGCTs, is a tumour entity with two distinct subtypes consisting of CS and SS. The CS is the common type in young men at the age of 18-50 (reviewed by Rørth et al. 2000). It is composed of fairly uniform medium-sized cells with clear cytoplasm and well-defined cell borders, resembling primitive germ cells. The cytoplasm is usually rich in glycogen and alkaline phosphotase. A characteristic feature of SE is the lymphocytic and granulomatous reaction of the stroma. The cells occur in lobules, rows or columns (Mostofi and Bresler 1976; Skakkebaek and Berthelsen 1978; Mostofi 1980; Skakkebaek et al. 1987).

The origin of CSs remains an enigma. Three hypotheses concerning the histogenesis of these tumours have been considered. Firstly, the CIS seems to originate from embryonal germ cells, the gonocytes. It may be possible that some germ cells survive in their embryonic state into adulthood (See Carcinoma *in situ*). Secondly, perhaps malignant transformation of type A spermatogonia is associated with the dedifferentiation of adult germ cells so that they revert structurally and functionally to more primitive precursor cells (Holstein et al. 1987). Thirdly, the most likely target cell type for transformation during germ cell development may be zygotene-pachytene spermatocytes with replicated chromosomes (Chaganti and Houldsworth 2000). The third hypothesis is based on the genetic properties of GCTs, *i.e.* increased 12p copy number, expression of cyclin D2 (CD2) in CIS, near triploid-tetraploid chromosome numbers, and abundant expression of

wild-type p53. CD2 is a chromosome 12p-based candidate gene whose overexpression may be induced by aberrant recombination of p53-arrested premeiotic pachytene spermatocytes. This gene acts as an oncogene via its ability to induce phosphorylation of the retinoblastoma gene product and could be responsible for stimulating the tetraploid cells, the arrested meiotic cells, to reinitiate the mitotic cycle (Chaganti and Houldsworth 2000).

The SS shows not only homogeneous fields or cords of cells, but also large, often gigantic cells that are supposed to represent spermatocytes or type B spermatogonia without the expression of PLAP, a differentiated spermatogenic cell type (Skakkebaek et al. 1987; Looijenga and Oosterhuis et al. 1999, Kraggerud et al. 1999).

1.2.4 Etiology and epidemiology of testicular germ cell tumours

Several predisposing factors for GCTs development have been identified, which include cryptorchidism, spermatogenic or testicular dysgenesis, Klinefelter's syndrome, prior history of a GCT or contralateral testis with GCT, and a positive family history (reviewed by Skakkebaek 1978; Dieckmann and Skakkebaek 1999; Rørth et al. 2000). Although the CIS cell is generally regarded as the precursor of adult male GCTs, the target stage of germ cell development at which transformation occurs is not known. It was suggested that foetal gonocytes have escaped properly timed differentiation (Skakkebaek et al. 1987), and undergo abnormal cell divisions mediated by a kit receptor/SCF paracrine loop leading to the origin of CIS cells (Skakkebaek et al. 1998; Rajpert-De Meyts et al. 1996b). The CIS cells give rise to both seminomatous and nonseminomatous GCTs (see Carcinoma *in situ*).

Abnormal function or a reduced number of foetal Sertoli cell and Leydig cells may be crucial, not only for abnormal preservation of gonocytes postnatally, but also for a parallel development of other gonadal abnormalities such as impaired spermatogenesis, undescended testis and hypospadias. Undescended testis, a common condition carrying a risk of testicular cancer, also seems to be associated with an increased number of gonocytes during the early postnatal years (Huff et al. 1993). Boys with cryptorchidism often harbour atypical spermatogonia with an aneuploid chromosome component (Müller and Skakkebaek 1984). A critical question is, however, which factors determine normal as opposed to abnormal differentiation of the gonocytes. In this regard, the Sertoli cells and Leydig cells and their products (growth factors and steroids) are interesting candidates. In other words, we should focus on the endocrinology and paracrinology within the testis.

The gonadal dysgenesis model poses an interesting paradox for the role of Sertoli cells in GCTs. On one hand, Sertoli cells in the dysgenetic testis may have been ineffective in stimulating the differentiation of the gonocytes into normal spermatogonia in foetal life, and are therefore responsible for the persistence of CIS-like cells. On the other hand the patients with gonadal dysgenesis often harbour a so-called gonadoblastoma, a tumour with low malignancy and invasiveness (Jørgensen et al. 1997). The germ cells of gonadoblastoma often have CIS characteristics: not only are they morphologically identical, but they also express immunohistochemical markers of CIS and have an

aneuploid DNA pattern similar to CIS cells (Jørgensen et al. 1997). Why do they tend to be clinically benign? The reason may be related to the lack of mitogenic stimuli from Sertoli cell-derived growth factors. The supporting cells of the gonadoblastoma cannot really be characterised as normal Sertoli cells.

Recent studies have shown that Leydig cell function may also be impaired in men with testicular cancer (Petersen et al. 1998). Androgen insensitivity syndrome is associated with a very high risk of germ cell neoplasia (Rørth et al. 2000). Is there an increasing trend of abnormalities of the male reproductive system including testicular cancer linked to increasing levels of estrogens during pregnancy? This question was recently posed by researchers interested in estrogen hypothesis, which implicates the so-called environmental estrogen as a possible cause for the increasing frequency of male reproductive abnormalities (reviewed by Toppari et al. 1996). Several studies suggested that some of the reproductive abnormalities to which testicular cancer is epidemiologically linked can be caused by exposure to increased levels of estrogen *in utero*. A high estrogen level in the pregnant woman could be a risk factor for development of testicular cancer in her son (Depue et al. 1983). Some evidence came from the investigation of offspring of mothers who were taking the potent estrogen diethylstilboestrol during pregnancy (Dieckmann et al. 1953). It was also demonstrated in a mouse model that intrauterine exposure to diethylstilboestrol could induce poor semen quality, cyptorchidism, and testicular cancer. However this model does not show the pathological characteristics of human SE and NSE (McLachlan et al. 1975; Newbold and Bunge 1987; Looijenga and Oosterhuis 1999). It is now clear that this is not the whole story. Late foetal life, postnatal, pubertal and post-pubertal endocrinological factors also seem to play a role (Toppari et al. 1996; Skakkebaek et al. 1998). Epidemiological evidence suggests that environmental factors are involved in the steadily increasing incidence of testicular cancer (Brown et al. 1987; Adami et al. 1994; Toppari et al. 1996; Skakkebaek et al. 1998). Many environmental pollutants have estrogenic or anti-estrogenic activity leading to the hypothesis that hormonal disruption may be causing the increased incidence of testicular cancer and genital abnormalities (reviewed by Toppari et al. 1996; Skakkebaek et al. 1998). Geographical differences in the incidence of testis cancer suggest that there are probably many confounding factors, including genetic susceptibility and lifestyle factors, such as diet.

One major reason for the poor understanding of the biology of GCTs is the lack of animal models and established cell lines. The establishment of a TGCT cell line or animal model may provide a powerful tool for studying the pathogenesis and novel treatment of TGCT. The first GCT cell line was established through the culture of EC cells (Fogh and Trempe 1975). Since then, various other NSE-TGCT cell lines have been established, and due to the stem cell features of these cells they can contribute to germ layers in the chimeric mice (Hanaoka et al. 1991). However, the establishment of SE cell lines can be very difficult because they cannot be cultured *in vitro* for a prolonged period (Berends et al. 1991; Olie et al. 1995). In 1995, Olie et al. were only able to initiate proliferation of SE cells and enhance the survival of the cell lines to a maximum of 30 days. In 1998, Tanaka's research group established the first SE cell line JKT-1, which has been cultured for more than two years and shows metastases when these cells are grafted to nude mice (Kinugawa et al. 1998). In 1999, this group isolated a new metastatic SE cell line JKT-

HM by transplanting cells from the cell line JKT-1 into the dorsal skin of nude mice. The transplanted cell line only forms SEs and the tumours are metastatic. JKT-HM displays the typical triploid karyotype of SE (Jo et al. 1999). Progress in this field of research has been hampered by the fact that no animal model has yet been established which mirrors testicular germ cell cancer in human.

1.2.5 Testicular tumours in animals

Spontaneous GCTs of the testis are extremely rare in rodents and other animals. Two cases of teratoma and EC in 8- and 10- week-old Sprague-Dawley IGS rats have recently been reported (Sawaki et al. 2000). Only a few cases of SE in animals have been published (Mitsumori et al. 1988; Veeramachaneni et al. 1999; Peters et al. 2000a,b, 2001). All the reported SE cases are invariably likely to be SS. The most common type of testis tumour in animals is of Leydig cell origin. The Leydig cell is a common target of compounds tested in rodent carcinogenicity bioassays (Cook et al. 1999). Although testicular tumours are inducible by endocrine disrupting chemicals (EDCs) such as diethylstilbestrol, most of the tumours are interstitial cell tumours, but not germ cell tumours (Imaida and Shirai 2000). Leydig cell and Sertoli cell tumours have also been studied in dogs (Peter et al. 2000a,b, 2001). In dogs with a SE, hormone concentrations were not different from those of normal dogs (Peters et al. 2000b). Dogs with a Sertoli cell or Leydig cell tumour had higher estradiol concentration and inhibin-like immunoreactivity in both peripheral and testicular venous circulation. Therefore it was concluded that SEs are not endocrinologically active (Peters et al. 2000b).

A possible murine model for human GCTs was identified early (Stevens and Little 1954; Stevens 1967): spontaneous testicular teratomas develop in the inbred mouse strain 129/Sv. In addition, testicular teratoma developed when genital ridges from day 12-13 embryos were transplanted into adult testis (Stevens 1970). These tumours have been studied extensively over the past four decades. They have been shown to be derived from PGCs, they comprise of apparently normal and mutated genomes, they are transplantable and able to participate in normal murine development (Matin et al. 1998). The human and this murine GCTs share certain features reflecting their germ cell origin, *i.e.* pluripotentiality, expression of wild type p53, and sensitivity to DNA-damaging agents (Matin et al. 1998; Lutzker and Levine 1996). These features have made them a potentially attractive model system for the analysis of carcinogenesis. Recent studies have shown that mutations at certain gene loci, such as Ter and Trp53, significantly enhance the susceptibility of the 129/Sv strain to develop teratomas (Asada et al. 1994; Donehower 1996). More recently, a 129 strain locus, a male germ cell tumour-susceptibility-determining locus pgct1 has been identified on chromosome 13 near D13Mit 188 (Muller et al. 2000). In contrast, adult human male GCTs arise from pre-existing CIS, their genomes are heteroploid and aberrant, and, as discussed above, no predisposing genetic factors have been identified as yet. Therefore, murine teratomas do not appear to be good models for adult male GCTs in terms of their ontogenesis.

1.3 GDNF family and receptors

1.3.1 Introduction to GDNF family and receptors

Glial cell line-derived neurotrophic factor (GDNF) family of neurotrophic factors consists of small secreted molecules that are responsible for many functions in the development, differentiation and survival of mainly neural but also other tissues (reviewed by Airaksinen et al. 1999; Saarma and Sariola 1999; Baloh et al. 2000). The first member of the GDNF family was identified due to the ability of the conditioned media of a glial cell line culture to promote the survival of dopaminergic neurones. GDNF was purified and cloned by this neuronal survival approach which is a classic way to search for secreted neurotrophic factors. The characterisation of GDNF rendered it to a distant member of the transforming growth factor β (TGFβ) family (Lin et al. 1993). New members of the GDNF family including neurturin (NRTN), persephin (PSPN) and Artemin (ARTN) were characterised thereafter (Kotzbauer et al. 1996; Baloh et al. 1998; Milbrandt et al. 1998). All these factors are structurally closely related sharing about 40% identity at amino acid level. They also share the sequence suggesting a common "cysteine knot" formed by three disulfide-bonds. Due to their function as disulfide-bonded homodimers and sharing of the conserved cysteine residues of TGFβ superfamily, they were classified as a subfamily of TGFβ superfamily, although the overall amino acid homology for instance to TGFβ-1 is not more than 20% (Milbrandt et al. 1998). Similar to neurotrophins, the GDNF family ligands are also first synthesised as precursor polypeptides that are processed to mature proteins and secreted (Lin et al. 1993) (Fig. 5).

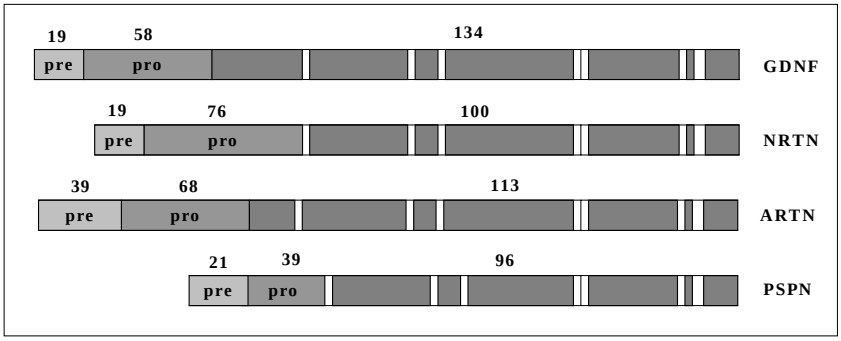

Figure 5. Schematic structure of the prepropeptides of GDNF family members. The number of amino acids of the pre- and proregions, and of mature proteins and the positions of the seven conserved cysteine residues (white lines) are shown. According to Airaksinen et al. 1999.

The potential receptor binding surfaces have been identified based on the X-ray crystal structure of GDNF (Eigenbrot and Gerber 1997). GDNF family members mediate their biological functions via a multicomponent receptor complex (see Fig. 6), which includes a signal transducing receptor, the Ret receptor tyrosine kinase, and

glycosylphosphatidylinositol (GPI)-linked cell surface coreceptors, GDNF family receptor alphas (GFRαs) (reviewed by Airaksinen et al. 1999; Saarma and Sariola 1999; Baloh et al. 2000; Takahashi 2001). The high affinity of GDNF binding to Ret is mediated through a GFRα receptor (originally named GDNF receptor-α) (Jing et al. 1996; Treanor et al. 1996). The primary specificity of the coreceptors for GDNF, NRTN, ARTN and PSPN is GFRα1, GFRα2, GFRα3, and GFRα4, respectively (reviewed by Airaksinen et al. 1999; Saarma and Sariola 1999; Baloh et al. 2000). However, a weak cross-talk between ligands (GDNF, NRTN, ARTN) and coreceptors (GFRα1, GFRα2, GFRα3) has been observed *in vitro* (Baloh et al. 1997; Jing et al. 1997; reviewed by Saarma 2000), except that mammalian GFRα4 receptor binds only PSPN (Lindahl et al. 2001). The downstream signalling pathways are thought to be mostly common for all GDNF family members since all GFRαs bind to and activate the same tyrosine kinase.

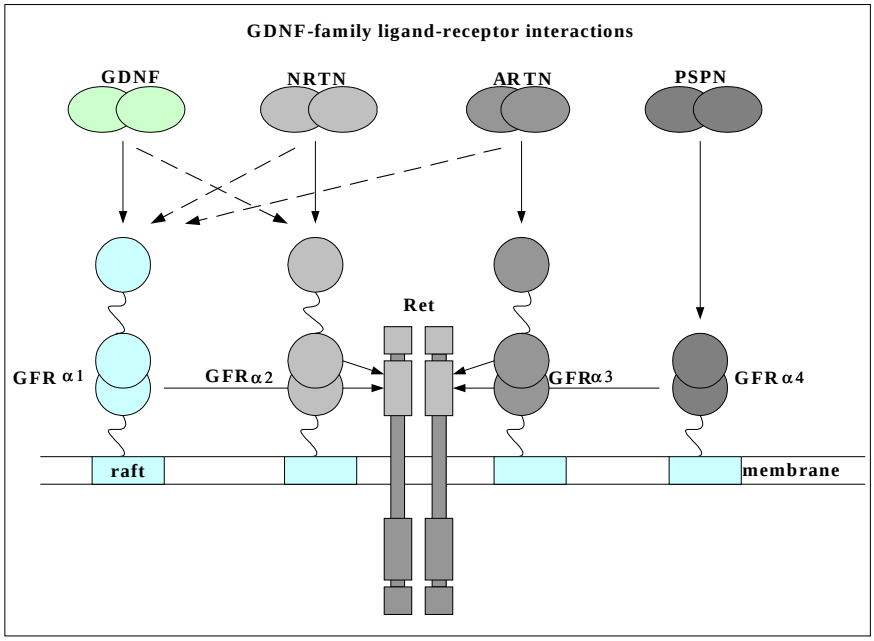

Figure 6. The binding of GDNF family ligands and receptors. The solid arrows represent the functional binding that activates Ret most potently, whereas the dotted arrows indicate weak interactions with receptor that may not be physiologically significant *in vivo.*

1.3.2 GDNF

Transcription of the GDNF gene usually produces a full-length GDNF mRNA of 4.5 kb, but a 6.0 kb transcript mRNA species has also been observed. The coding region of GDNF gene is 633 bp, which encodes a 211 amino acid precursor polypeptide, from which the mature protein of 134 amino acid is produced by proteolytic cleavage (Lin et

al. 1993). There are two potential glycosylation sites in the mature protein, which has a Mr of 18 to 22 kDa (Lin et al. 1993). An alternatively spliced GDNF mRNA lacking 78 bp in the preproregion has been found, but since it gives rise to the same mature GDNF, the function of the different splicing is unknown (Suter-Crazzolara and Unsicker 1994).

GDNF promotes the survival of several types of neurones in both the central and peripheral nervous systems. Exogenous GDNF can maintain dopaminergic, noradrenergic and motor neurones of the central nervous system (Lin et al. 1993; Henderson et al. 1994; Arenas et al. 1995; Oppenheim et al. 1995; Tomac et al. 1995; Yan et al. 1995; Gash et al. 1996; Messer et al. 2000; Rosenblad et al. 2000), as well as various sub-populations of the peripheral sensory and sympathetic neurones (Henderson et al. 1994; Buj-Bello et al. 1995; Ebendal et al. 1995; Trupp et al. 1995; Arce et al. 1998). The pharmacological effects of GDNF on midbrain dopaminergic neurones have been studied on several animal models in both rodents and non-human primates (reviewed by Lapchak et al. 1997; Grondin et al. 1998). Therefore, GDNF is considered a potential drug candidate for the treatment of Parkinson's disease. The *in vivo* functions of GDNF in the central and peripheral nervous systems have been studied by the *in vivo* loss-of-function and gain-of-function of GDNF in transgenic models, (Moore et al. 1996; Pichel et al. 1996; Sánchez et al. 1996; Nguyen et al. 1998). The development of the dopaminergic neurones in GDNF-deficient embryos is normal. However the homozygous mice die at birth before the maturation of dopaminergic nervous system. The GDNF-deficient mice show a 20%-30% reduction in the number of motoneurones in trigeminal ganglia and the spinal cord (Moore et al. 1996; Sánchez et al. 1996). The transgenic mice overexpressing GDNF under a muscle-specific myogenin promoter show hyperinnervation of neuromuscular junctions for several weeks after birth (Nguyen et al. 1998), suggesting that GDNF also functions as a muscle-derived factor for motor axon branching and synapse elimination. The studies of GDNF-/-, GFRα1-/-, and Ret-/- mice revealed that these mutant mice had a remarkably similar phenotype. These mutant mice fail to develop kidneys and to innervate the gastrointestinal tract below the stomach (Schuchardt et al. 1994, 1996; Moore et al. 1996; Pichel et al. 1996; Sánchez et al. 1996; Cacalano et al. 1998; Enomoto et al. 1998). Subsequent analyses of the enteric nervous system in these mice establish that GDNF is crucial for the early development of the enteric nervous system (Taraviras et al. 1999). A loss of certain cranial parasympathetic ganglia (otic and sphenopalatine) of the newborn Ret-, GFRα1-, and GDNF-mutants has been observed indicating that GDNF has a similar role in the early development of the parasympathetic nervous system (Marcos and Pachnis 1996; Enomoto et al. 2000; Rossi et al. 2000).

Outside the nervous system, GDNF mRNA is abundantly expressed in the condensating nephrogenic mesenchyme cells around the tips of the invading ureteric buds while branching bud tips express the functional receptor Ret (Pachnis et al. 1993; Hellmich et al. 1996; Suvanto et al. 1996; Widenfalk et al. 1997, 2000). The development of the metanephric kidney is initiated by the reciprocal interaction of nephrogenic mesenchyme and the Wolffian duct-derived ureteric bud (Saxén 1987). This inductive interaction of the mesenchyme and ureteric epithelium can be mediated by growth factors and their receptors (Saxén 1987; Sariola and Sainio 1997; Sariola and Saarma 1999). The special expression pattern of GDNF and Ret suggested a crucial paracrine regulatory role of

GDNF in kidney morphogenesis. *In vitro* culture studies and gene disruption studies clarified that GDNF, a mesenchyme-derived signal, is crucial in the regulation of ureteric bud epithelia in branching morphogenesis (Durbec et al. 1996a; Sainio et al. 1997; Pepicelli et al. 1997). GDNF-deficient mice lack kidneys and die in the first postnatal day (Moore et al. 1996; Pichel et al. 1996; Sánchez et al. 1996). GDNF haploinsufficiency in heterozygotes (GDNF+/-) of these mice often exhibits unilateral renal agenesis or rarely dysgenesis (Moore et al. 1996; Pichel et al. 1996; Sánchez et al. 1996). *In vitro*, early ureteric buds will grow toward localised sources of GDNF (Durbec et al. 1996a; Pepicelli et al. 1997; Sainio et al. 1997). GDNF increases cell mobility, dissociation of cell adhesion, and migration of Ret-transfected Madin-Darby canine kidney (MDCK) cell line toward a localised source of GDNF (Tang et al. 1998). Disruption of GDNF function with neutralising antibodies also showed that GDNF is necessary for the development of the ureteric bud (Vega et al. 1996). Addition of soluble GDNF to organ culture results in an increased number of ureteric tips (Vega et al. 1996, Pepicelli et al. 1997). The inductive signals may be mediated by GDNF through its receptor without the close interaction of mesenchyme and epithelia. It has been recently shown that GDNF, combined with mesenchyme-derived soluble factors, also induces branching morphogenesis of ureteric buds in the absence of mesenchymal tissue (Qiao et al. 1999). One candidate for the unknown metanephric mesenchymal derived soluble factor(s) was recently identified as pleiotrophin (Sakurai et al. 2001). It has also been proposed that an unknown stromal signal is induced by retinoic acid (Vitamin A), which then induces Ret expression in the ureteric bud (Batourina 2001).

During development, GDNF is abundantly expressed in a number of tissues where the possible role of GDNF is still unclear. For instance, GDNF is expressed in the mesenchyme of the developing limb, in the tip of the embryonic tongue, cartilage and derivatives of the pharyngeal pouches (Hellmich et al. 1996; Suvanto et al. 1996; Golden et al. 1999). GDNF is also expressed in the testis and probably by Sertoli cells (Hellmich et al. 1996; Suvanto et al. 1996; Golden et al. 1999; Viglietto et al. 2000; Widenfalk et al. 2000). GDNF is able to stimulate mitosis of cultured germ cells isolated from prepuberal mice (Viglietto et al. 2000), and in postnatal rat testes, GDNF was demonstrated to enhance FSH-induced Sertoli cell and gonocyte proliferation (Hu et al. 1999).

1.3.3 NRTN

NRTN, with 42% similarity to GDNF at amino acid level, was identified on the basis of its ability to support the survival of sympathetic postganglionic neurones in culture (Kotzbauer et al. 1996). Similar to GDNF, NRTN is first synthesised as a precursor polypeptide, then processed to mature protein, and secreted (Fig. 5). The mature NRTN has a predicted Mr of 11.5 kDa (Kotzbauer et al. 1996).

NRTN promotes the survival of several populations of neurones in both the central and peripheral nervous systems (Klein et al. 1997). Like GDNF, NRTN also has a survival-promoting effect on dopaminergic neurones *in vitro* (Horger et al. 1998). *In vivo*, NRTN has been shown to exert neuroprotective effects in the 6-hydroxydopamine (6-OHDA) lesion model (Horher et al. 1998; Rosenblad et al. 1999a,b). There is a difference between the effect of GDNF and NRTN on developing and adult substantia nigra

dopaminergic neurones; GDNF has wider survival, neuritogenic and hypertrophic effects as compared to the selective survival-promoting effects of NRTN (Åkerud et al. 1999; Rosenblad et al. 1999a,b). NRTN affects parasympathetic neuronal survival and target innervation. NRTN-deficient mice have a strikingly similar phenotype with the mice lacking GFRα2 showing defects in the parasympathetic enteric nervous system (Heuckeroth et al. 1999; Rossi et al. 1999). They are viable and fertile, but have a dramatically reduced parasympathetic cholinergic innervation of the lacrimal and salivary glands, and a reduced myenteric plexus innervation. NRTN and GFRα2 together with Ret regulate the parasympathetic innervation of the penis and may also have a role in the regulation of heart innervation (Laurikainen et al. 2000; Hiltunen et al. 2000).

During embryogenesis the mRNAs for Ret, GFRα1, and GFRα2 are colocalised in cranial parasympathetic ganglia (Golden et al. 1999; Enomoto et al. 2000; Rossi et al. 2000). Embryonic submandibular ganglia neurones respond to NRTN and GDNF, even in the absence of GFRα1 suggesting some cross-talk between GDNF and GFRα2 (Cacalano et al. 1998). GDNF and NRTN have distinct roles in the regulation of cranial parasympathetic ganglia *in vivo*. GDNF signalling via GFRα1/Ret is essential for gangliogenesis of some cranial parasympathetic neurones, whereas subsequent NTRN signalling through GFRα2/Ret is crucial for the development and maintenance of parasympathetic target innervation (Rossi et al. 2000).

NRTN is widely expressed in other non-neuronal cells and tissues (Widenfalk et al. 1997; Golden et al. 1999), where its role remains unclear. In the embryonic kidney, NRTN is expressed in both the mesenchyme and developing bud epithelium where it colocalises with Ret (Widenfalk et al. 1997). The expression patterns of NRTN and Ret therefore suggests a local autocrine regulation in epithelial development of the ureteric bud. NRTN is capable of promoting ureteric bud branching (Davies et al. 1999). However, NRTN-deficient mice do not show any defects in kidney morphogenesis, suggesting either that NRTN might be unimportant for kidney development or a possible functional redundancy. Although both NRTN and GFRα2 are highly expressed in the testis (Widenfalk et al. 1997, 2000; Golden et al. 1999; Viglietto et al. 2000) and both GDNF and NRTN were able to stimulate mitosis of cultured germ cells isolated from prepuberal mice (Viglietto et al. 2000), no testis phenotype has yet been reported from both NRTN- and GFRα2-deficient mice.

1.3.4 Ret receptor tyrosine kinase

1.3.4.1 Ret is a functional receptor for GDNF family ligands

The Ret (**re**arranged during **t**ransfection) proto-oncogene was first identified by Takahashi and co-workers who found a novel gene rearrangement and oncogenic activity in transfected NIH 3T3 cell line. The gene encodes a novel tyrosine kinase that has an extracellular ligand binding domain with four cadherin-like repeats and a cysteine-rich domain, a hydrophobic transmembrane region and a cytoplasmic domain with an intrinsic tyrosine kinase activity (Takahashi et al. 1985; Takahashi and Cooper 1987; Anders et al. 2001). In the extracellular domain the cysteines are probably involved in the formation of

disulfide bonds, allowing the acquisition of the correct secondary structure (Ponder 1999). Three isoforms of Ret are generated by alternative splicing at the 3' end of the gene. The long, intermediate, and short Ret isoforms which differ by 51, 43, and 9 amino acids in the C-terminus are referred to as Ret51, Ret43, and Ret9. Two major isforms Ret51 and Ret9 have different signalling properties *in vivo* (de Graaff et al. 2001). The extracellular portion of the molecule contains several glycosylation sites. Western blot analysis shows two Ret isoforms of *M*r 150 kDa and 170 kDa that are glycosylated differently (reviewed by Ponder 1999). The *M*r 170 kDa isoform is the fully mature version of the receptor and is present on the cell membrane. The *M*r 150 kDa isoform is a maturation intermediate that is present only in the endoplasmic reticulum (reviewed by Ponder 1999).

The ligand for the orphan receptor Ret remained unknown for years until it was discovered by several groups that the Ret tyrosine kinase is the functional receptor for GDNF (Durbec et al. 1996a; Trupp et al. 1996; Jing et al. 1996; Treanor et al. 1996). Thereafter, Ret was also shown to be the functional receptor for other members of the GDNF family (reviewed by Airaksinen et al. 1999; Saarma and Sariola 1999; Baloh et al. 2000; Saarma 2000). Ret is expressed in several tissues overlapping with or adjacent to sites of GDNF family ligands. During embryonic development, the Ret gene is highly expressed in parts of the central nervous system, in the neural crest and many of its derivatives, including the peripheral nervous system and neuroendocrine cells, such as the C-cells of the thyroid and chromaffin cells of the adrenal gland (Pachnis et al. 1993; Takahashi et al. 1993; Tsuzuki et al. 1995; Watanabe et al. 1997). *In vivo* data from gene knockout studies give further evidence for the interaction of GDNF and Ret, because GDNF and Ret null mice show a strikingly similar phenotype. The homozygous mice die during 24 hours after birth due to renal aplasia or hypoplasia, and lack of enteric innervation (Schuchardt et al. 1994, 1996). The discrepancy of some phenotypes between Ret and GDNF null mice, for instance the less severe loss of superior cervical ganglia in GDNF null mice, may be due to the functional compensation of GDNF by other factors.

Histoarchitecture of the mature kidney is determined largely by the pattern of growth and branching of the ureteric bud (Saxén 1987). In the developing kidney the striking parallel expression of Ret and GDNF has been observed (Pachnis et al. 1993; Tsuzuki et al. 1995; Suvanto et al. 1996; Sariola and Sainio 1997). The restricted expression of Ret and GDNF might be important in regulating the extent, and possibly pattern, of ureteric bud growth and branching (Sariola and Sainio 1997, Sariola and Saarma 1999; Srinivas et al. 1999). The misexpression of Ret throughout the ureteric bud branches under the Hox7 promoter, in contrast to the normal expression only at the bud tip, causes variable inhibition of ureteric bud growth and branching reminiscent of, but less severe than, the Ret-deficient phenotype (Srinivas et al. 1999). Moreover, expression of a Hox7/Ret-PCT2 transgene, encoding a ligand-independent form of Ret kinase, also causes the development of abnormal nodules, outside the kidney or at its periphery, containing branched epithelial tubules apparently formed by deregulated growth of the ureteric bud. This suggested that Ret signalling is not only necessary but also is sufficient to induce ureteric bud growth, and that the orderly, centripetal growth of the bud tips is controlled by the spatially and temporally regulated expression of GDNF and Ret (Srinivas et al. 1999).

1.3.4.2 Ret gene in cancer and Hirschsprung's disease

After the Ret proto-oncogene was discovered, various oncogenic mutations and translocations of the proto-oncogene were soon found. Gain-of-function and loss-of-function mutations in the Ret gene lead to cancer syndromes or Hirschsprung's disease (HSCR), respectively. Ret induces cell transformation when translocated (Takahashi et al. 1985). Such gene translocations have been found in human papillary thyroid carcinoma (PTC) (Grieco et al. 1990), where the tyrosine kinase domain of the Ret gene is fused with the 5' sequence of a gene that is expressed in follicular cells leading to ectopic Ret activation. Activating Ret mutations have been described in the multiple endocrine neoplasia type 2 (MEN 2) hereditary cancer syndromes (reviewed by Eng 1999; Ponder 1999; Takahashi 2001). MEN 2 are dominantly inherited syndromes that comprise of three clinical subtypes: MEN 2A, MEN 2B, and familial medullary thyroid carcinoma (FMTC). All three include the C-cell tumours, medullary thyroid carcinomas (MTCs), which are more aggressive in patient with MEN2B than those seen in patients with FMTC or MEN 2A. MEN 2A is characterised by the association with MTC, pheochromocytomas, and hyperparathyroidism, while MEN 2B includes MTC, pheochromocytomas, neuromas of the lips, tongue and conjunctivae, intestinal ganglioneuromatosis, marfanoid skeletal abnormalities and a male reproductive defect due to erectile dysfunction with a neurological basis (reviewed by Pasini et al. 1996; Eng 1999; Ponder 1999). In FMTC, the MTC is the sole clinical manifestation and develops late in life (reviewed by Eng 1999; Ponder 1999). All affected patients in MEN 2A and 2B develop MTC in childhood or as teenagers. In sporadic MTC, tumours are usually single and unilateral. In patients with MEN 2A, MEN 2B, and FMTC, the tumour occurs as a multifocal, bilateral disease.

The majority of the MEN 2A and FMTC mutations are located in the extracellular domain. Missense mutations at one of five cysteines clustered in the extra-cytoplasmic domain of Ret (Cys 609, 611, 618, 620, and 634) have been identified in the majority of MEN 2A families and in approximately two-thirds of FMTCs (reviewed by Edery et al. 1997; Eng 1999; Blume-Jensen and Hunter 2001; Manié et al. 2001). These alterations result in the substitution of a single cysteine by a different amino acid causing constitutive signalling of the tyrosine kinase through the formation of disulfide-linked Ret homodimers (reviewed by Ponder 1999; Takahashi 2001). The great majority of MEN 2A mutations affect codon 634, while in FMTC the mutations are equally distributed among codons 618, 620, and 634. Approximately 95% of MEN 2B cases as well as most cases of spontaneous MTC, result from a substitution of threonine for methionine at codon 918 within the catalytic core of the Ret kinase domain. This replacement increases the kinase activity of Ret without constitutive dimer formation, most likely because it enables substrate access without prior autophosphorylation of the active loop (reviewed by Ponder 1999; Takahashi 2001). In addition, the Ret mutation in MEN 2B alters the substrate specificity of the Ret/MEN 2B receptor towards peptide substrates that are optimal for Src and Abl. The altered substrate specificity of Ret MEN 2B may contribute to the aggressiveness of MEN 2B *in vivo* (reviewed by Blume-Jensen and Hunter 2001). There is a significant increase in phosphorylation after the GDNF stimulation of the mutant Ret-MEN 2B *in vitro* (Bongarzone et al. 1998). The resulting combined

oncogenic activation is certainly stronger than that provided by the Cys-634 mutation. The long Ret isoform with the MEN 2B mutation is approximately two-fold more transforming *in vitro* than the same Ret isoform with a MEN 2A mutation (Cys 634 to Arg). This could be an additional explanation for the earlier onset of tumours in MEN 2B (Iwashita et al. 1996).

In all these hereditary or sporadic malignant diseases, Ret is constitutively phosphorylated due to missense mutations either in the extracellular domain, critical for the receptor dimerisation, or in the intracellular catalytic domains of the tyrosine kinase. Interestingly, the tumours in the MEN 2 develop in only a few organs, although Ret is expressed in many tissues, suggesting modulatory mechanisms, which are supposed to be mediated by the different GFRα receptors (Kawai et al. 2000, Lindahl et al. 2001). Recently, receptors Ret, GFRα1 and 2, and ligands (GDNF and NRTN) were found in seminoma cells (Viglietto et al. 2000), but it is not clear yet if Ret is mutated or hyperphosphorylated in seminoma or has a role in its pathogenesis.

In humans, heterozygous loss-of-function mutations in Ret lead to absence of enteric ganglia from the distal colon and congenital megacolon (HSCR; Parisi and Kapure 2000). These mutations vary from missense or nonsense point mutations to deletions. They result in the inactivation of the tyrosine kinase or ligand binding domain or in the perturbance of the membrane transport of the receptor (reviewed by Iwashita et al. 2001, Manié et al. 2001, Takahashi 2001). In contrast, inactivation of Ret in mice causes severe intestinal agangliosis (Schuchardt et al. 1994, 1996; Durbec et al. 1996b). NRTN and GDNF promote proliferation, survival and differentiation of enteric neurones and glial progenitors *in vitro*, GDNF also promotes the migration of enteric crest cells which is believed to be an important cause in HSCR (Heuckroth et al. 1998; Worley et al. 2000; Taraviras 1999; Young 2001). Though some of the cellular processes controlled by Ret (such as survival, migration, and differentiation of enteric neuron cells) have been identified in animal models, the mechanisms that lead to the localised absence of enteric ganglia in HSCR patients remain unclear.

1.3.4.3 Oncogenic Ret mutations in transgenic mice models

The Ret/PTC oncogene, a rearranged form of the Ret proto-oncogene, has been found in human PTCs. Transgenic mouse models with PTCs have been generated by several research groups (Jhiang et al. 1996, 1998; Santoro et al. 1996; Portella et al. 1996; Sagartz et al. 1997; Powell et al. 1998; Cho et al. 1999). Transgenic mice with thyroid-specific expression of the Ret/PTC1 oncogene develop thyroid carcinomas with considerable similarities to human PTCs, particularly in the nuclear cytological features and the presence of local invasion (Jhiang et al. 1996; Santoro et al. 1996). The thyroid tumours in these Ret/PTC1 transgenic mice are characterised by a slow growth rate, thyroid-stimulating hormone-responsive tumour progression, and loss of radioiodide-concentrating activity despite continued expression of thyroglobulin (Sagartz et al. 1997; Jhiang et al. 1998). The time of the tumour onset appears to be dependent on the expression levels of Ret/PTC1 in the transgenic mice (Jhiang et al. 1998; Cho et al. 1999). The tumours induced by Ret/PTC3 are more aggressive than tumours induced by

Ret/PTC1, and metastatic solid-type papillary carcinomas are formed (Powell et al. 1998). Interestingly, the ubiquitous expression of Ret/PTC1 in transgenic mice carrying the Ret/PTC1 under the H4 promoter only develop mammary and cutaneous gland tumours, suggesting that Ret/PTC1 is able to couple with transforming pathways specific for these glandular cells (Portella et al. 1996).

All MEN 2 mutations tested so far convert the Ret proto-oncogene into a dominantly acting transforming gene, as the wild-type Ret allele is usually retained and expressed in tumour tissue (Tsuzuki et al. 1995). Ret-MEN 2A and Ret-MEN 2B mutations are oncogenic in the fibroblast transformation assay (Rossel et al. 1997). Their biological effects in thyroid C-cells and adrenal chromaffin cells have remained elusive. It was not clear whether these mutations interfered with one or more normal developmental functions of Ret. Therefore, transgenic models have been created to solve these questions.

The most common mutation in MEN 2A (52% of all cases) occurs at codon 634 and results in substitution of Cys-634 for an Arg (TGC→CGC). To examine whether expression of a MEN 2A allele of *Ret* results in transformation of C cells, transgenic mice have been constructed in which an active MEN 2A-Ret cDNA is expressed under a calcitonin/calcitonin gene-related peptide promoter, giving strong expression in thyroid C cells. The mice develop bilateral multiple foci of MTC, similar to the condition seen in the thyroid medulla of human carriers of a MEN 2A mutation (Michiels et al. 1997). However, the ectopic expression of the 634Arg-Ret transgene in several other tissues, such as in the kidney and brain which are not affected in the human MEN 2A, did not cause any histological abnormalities suggesting tissue-specific control of carcinogenesis. The tissue-specific carcinogenesis was also observed recently in transgenic mice ubiquitously expressing the MEN 2A-Ret oncogene fused to Moloney murine leukaemia virus long terminal repeats (Kawai et al. 2000). Expression of the transgene was detected at variable levels in a variety of tissues including the thyroid, heart, liver, colon, parotid gland, testis and brain. All mice developed thyroid C-cell hyperplasia or medullary carcinoma, accompanying high levels of serum calcitonin. In addition, the development of mammary or parotid gland adenocarcinoma was observed in half of the mice. Ret dimerisation and complex formation with Shc and grb2 adaptor proteins were detected in tumour tissues. Unexpectedly, no tumour formation was found in other tissues and receptor dimerisation was undetectable, although Ret-MEN2A expression was detected.

Most cases of MEN 2B are caused by substitution of Thr for Met918 in the Ret receptor tyrosine kinase. When the MEN 2B mutant Ret gene was introduced into the thyroid C cells, the transgenic mice developed MTC (Acton et al. 2000). The expression of MEN2B in various tissues, including the brain, lung, and kidney, did not induce tumours or developmental abnormalities. Transgenic mice were created using the dopamine β-hydroxylase (DβH) promoter to direct expression of MEN 2B-Ret to the developing sympathetic and enteric nervous systems and adrenal medulla. In DβH-MEN 2B-Ret mice, MEN 2B-Ret expression did not affect terminal innervation or induce intestinal ganglioneuromas or pheochromocytomas (Sweetser et al. 1999). Only sympathetic ganglioneuromas and renal anomalies are induced in these transgenic mice, and the renal abnormalities may due to the hyperplasia of sympathoadrenal ganglia that could compete

for GDNF binding with the ureteric bud (Sweetser et al. 1999; Gestblom et al. 2000). Recently, a new MEN 2B-Ret mouse strain has been generated by introduction of the corresponding mutation (Ret-Met919Thr) into the Ret locus using gene-targeting techniques in conjunction with Cre/loxP site-specific recombination (Smith-Hicks et al. 2000). Mutant mice displayed C-cell and chromaffin cell hyperplasia, the latter progressing to pheochromocytoma. Homozygotes did not develop gastrointestinal ganglioneuromas, but displayed ganglioneuromas of the adrenal medulla, enlargement of the associated sympathetic ganglia and male infertility. Surprisingly, homozygotes did not display any developmental defects attributable to a loss-of-function mutation. Thus, the results support the conclusion that the Met918 substitution is responsible for MEN 2B and that the altered substrate specificity of the mutant Ret kinase does not interfere with its normal role in the development of the kidneys and enteric nervous system (Smith-Hicks et al. 2000). The development of tumours in MEN 2B transgenic mice has previously been postulated to be sensitive to gene dosage. In contrast to MEN 2B knock-in mice, the MEN 2B-Ret overexpressing mice harbour a multiple transgene under the control of a heterologous promoter, which may account for the difference in the development of MTC.

1.3.5 GDNF signalling

1.3.5.1 Ret-dependent GDNF family signalling

GDNF family members bind to specific GFRα co-receptors, but all of them activate Ret. Ret is unable to bind GDNF on its own but can be activated in a complex with GFRα1 (Jing et al. 1996; Treanor et al. 1996). Other members of the GDNF ligand family bind to Ret with the aid of different members of (GFRα1-4) of the GFRα family of GPI-liked coreceptors (reviewed by Airaksinen et al. 1999). The lipid rafts are membrane structures of sphingolipids and cholesterol packed into moving platforms within the lipid bilayer. Raft microdomains may help to compartmentalise sets of signalling molecule at both sides of the plasma membrane, allowing them to interact with each other in a regulated manner, and at the same time preventing them from interacting with proteins excluded from rafts (Simons and Toomre 2000). It was discovered that inactive Ret is localised outside rafts (Paratcha et al. 2001, reviewed by Saarma 2001). Furthermore, GDNF signal transduction is dependent on intact rafts, because cholesterol depletion with methyl-β-cyclodextrin decreases GDNF signalling. Current experimental data favour the existence of two models for the Ret activation including *in cis* signalling and *in trans* signalling mechanisms. In cells coexpressing Ret and GFRα1, which is located in lipid rafts (Poteryaev et al. 1999; Trupp et al. 1999), GDNF induces and/or stabilises the formation of a complex between the two receptors, then interacts with two molecules of Ret and induces their homodimerisation and autophosphorylation at tyrosine residues (*in cis* signalling, see Fig. 7) (Paratcha et al. 2001; reviewed by Saarma 2001). GFRα1 recruits Ret to lipid rafts only following GDNF stimulation, resulting in the association of Ret and Src.

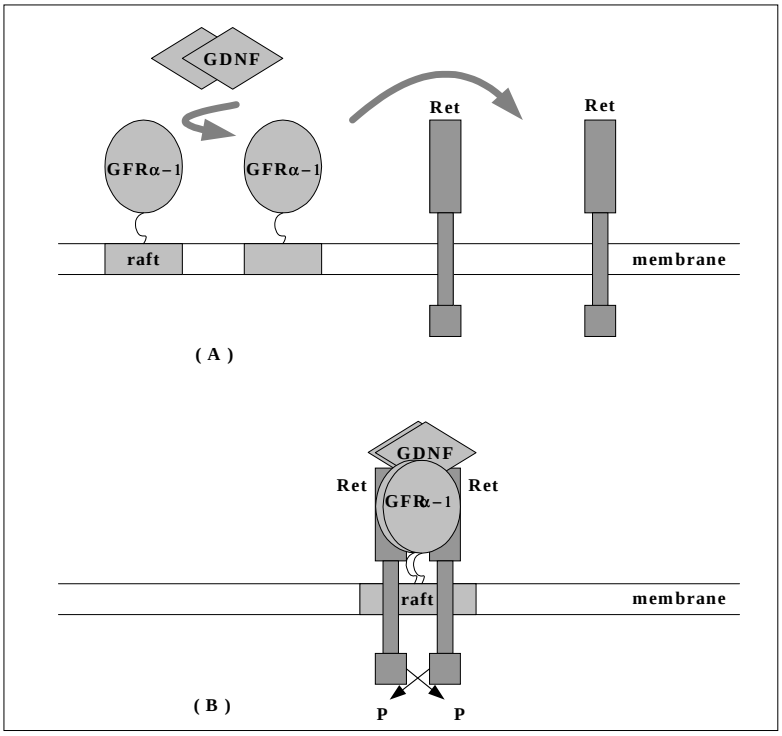

Figure 7. Schematic presentation of the GFRα1 inducing Ret homodimerisation *in cis* and autophosphorylation at tyrosine residues.

The overlapping and complementary expression of GFRαs with each other, with ligands, and with Ret, suggests that the functions of the GDNF family can be regulated in various ways. GFRα1 is widely expressed in the absence of Ret, suggesting alternative roles for "ectopic" sites of GFRα1 expression. GFRα1 also binds its ligand and activates Ret when provided exogenously in soluble form or immobilised on agarose beads (Treanor et al. 1996; Yu et al. 1998; Qiao et al. 1999). GFRαs are usually bound to the plasma membrane but a specific cleavage by a putative phospholipase or protease produces soluble forms of these co-receptor (Paratcha et al. 2001). Thus, another possibility is that GFRα1 may also function in a non-cell-autonomous manner to capture and concentrate diffusible GDNF family ligands from the extracellular space and then present these factors *in trans* to affect Ret-expressing cells (*in trans* signalling, see Fig. 8) (Paratcha et al. 2001; Saarma 2001). Activation of Ret *in trans* also results in the mobilisation of Ret to lipid raft membranes even in cells that lack endogenous GPI-anchored GFRα1 (Paratcha et al. 2001). Upon activation *in cis*, Ret is recruited to membrane rafts by a rapid extracellular mechanism, likely driven by its affinity for newly formed GDNF/ GFRα1 complexes, independently of its tyrosine kinase activity. In contrast, recruitment *in trans* is delayed, sustained, and requires an active Ret tyrosine kinase, suggesting the

involvement of intracellular events (Paratcha et al. 2001). Downstream signalling after stimulation *in trans* is at least as efficient as that induced *in cis* and potentiates downstream signalling, neuronal survival and differentiation (Paratcha et al. 2001).

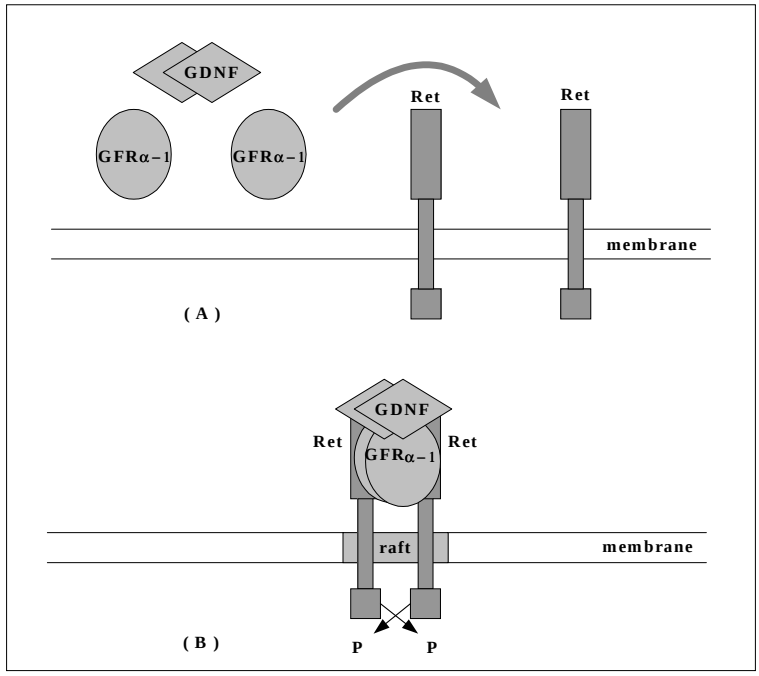

Figure 8. Schematic presentation of GFRα1 inducing Ret homodimerisation *in trans* and autophosphorylation at tyrosine residues.

Based on the existing data it is assumed that also other members of the GDNF family, NRTN, ARTN and PSPN interact with their cognate co-receptor and activate Ret similarly to GDNF.

1.3.5.2 The downstream pathways of Ret signal transduction

Since Ret was discovered as the functional receptor for GDNF, the intracellular signalling through Ret has been extensively studied (reviewed by Airaksinen et al. 1999; Hayashi et al. 2000; Takahashi 2001). Like other tyrosine kinases, upon ligand binding, Ret can activate various signalling pathways including RAS/extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)/AKT, p38 mitogen activated protein kinase (MAPK) and c-Jun N-ternimal kinase (JNK) pathways (See Fig. 9; Airaksinen et al. 1999; Trupp et al. 1999; Hayashi et al. 2000; Takahashi et al. 2001). It is well known that these signals are transmitted via phosphotyrosines present in the intracellular domains of activated receptors. Ret autophosphorylates its tyrosine residues Tyr-905, Tyr-1015, Tyr-1062 and Tyr1096, which serve as docking sites for the transduction effectors GRB10,

phospholipase Cγ (PLCγ), SHC/SNT (FRS2)/Enigma, and GRB2, respectively (reviewed by Hayashi 2000; Takahashi 2001). The former three tyrosines are conserved in all three Ret isoforms but tyrosine 1096 is present in the long isoform only. Among these Tyr residues, Tyr 1062 has a critical role in signalling initiation during embryogenesis and tumorigenesis, serving as a docking site for protein complexes (reviewed by Hayashi et al. 2000; Takahashi 2001). All of the RAS/ERK, PI3K/AKT, p38MAPK and JNK pathways are activated mainly through tyrosine 1062 that is a binding site for SHC adaptor proteins (reviewed by Hayashi et al. 2000; Takahashi 2001). After binding of SHC to tyrosine 1062, SHC further associates with GAB1/2 adaptor proteins and GRB2/SOS complex, leading to the activation of PI3K/AKT and RAS/ERK signalling pathways, respectively. SNT/FRS2 is a lipid anchored docking protein with a phosphotyrosine-binding (PTB) domain, and it may be involved mainly in activation of the RAS/ERK signalling pathway. The binding affinity of SHC and SNT/FRS2 to Ret was different among the three isoforms, their binding affinity to the middle isoform Ret was significant lower compared to the short and long isoforms. The p38MAPK, JNK pathways are also activated through tyrosine 1062. Phosphopeptide maps have showed that the autophosphorylation of Ret, found in MEN 2B, differs from that of the wild type and MEN 2A-Ret protein suggesting that also their downstream targets may differ (Liu et al. 1996). Some of these targets have now been identified (Bocciardi et al. 1997).

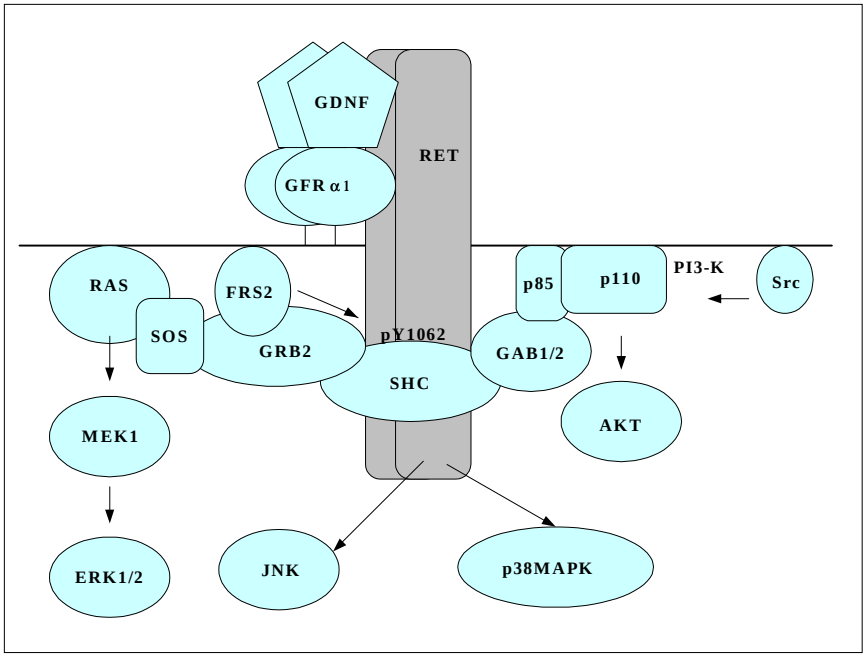

Figure 9. Schematic presentation of known or proposed intracellular Ret-binding proteins. Ret activates several intracellular pathways typical of receptor tyrosine kinase

signalling, including Ras-MAPK, phosphoinositol-3-kinase (PI3-K), Jun N-terminal kinase (JNK), and p38MAPK. According to Hayashi et al. 2000 and Takahashi 2001.

1.3.5.3 Ret-independent GFRα1 signalling

Recently it was found that a Ret-independent pathway of GDNF signalling may occur in lipid rafts (Poteryaev et al. 1999; Trupp et al. 1999). In cultured dorsal root ganglia neurones isolated from E18 Ret-deficient mice, GDNF can induce a rapid activation of Src-type kinases as well as MAPK (Poteryaev et al. 1999). Soluble GFRα1, which does not have GPI-anchors, cannot transduce the signal in response to GDNF binding in Ret-deficient cells. Therefore lipid rafts are believed to play a key role in Ret-independent signalling. It remains unknown whether this has relevance *in vivo* and whether other tyrosine kinase receptors, instead of Ret, are involved in the Ret-independent GFRα signalling.

2. AIMS OF THE STUDY

The aims of this thesis were to study the biology and pathology of GDNF family signalling in spermatogenesis and pathogenesis of testicular tumours. In particular, to compare the different roles of GDNF and NRTN in spermatogenesis and tumour development.

The specific aims were:

- To study the role of GDNF and NRTN in spermatogenesis.
- To clarify the properties and developmental features of the spermatogonial cells arrested by the overexpression of GDNF.
- To characterise the GDNF-induced testis tumours and study the pathogenetic role of GDNF signalling in seminomatous GCTs.
- To study the downstream molecular mechanisms mediating the biological and pathological functions of GDNF in the testis.

3. MATERIALS AND METHODS

3.1 DNA constructs and production of transgenic mice

3.1.1 pEFBOS-hGDNF mice and pEFBOS-NRTN mice

Full-length human GDNF (Genebank accession number: L15306) was cloned into the *Xba*I site of the PEF-BOS vector under the human EF-1α promoter in the sense orientation (Mizushima and Nagata 1990). The correctness of the construct was verified by sequencing, and GDNF expression was verified by Cos cell transfection and Western blotting. Transgenic mice were produced by microinjecting the 2.7 kb *Pvu*I-*Hind*III fragment of the construct, which contains the EF-1α, hGDNF insert and polyadenylation signal, into the pronuclei of newly fertilised FVB mouse eggs as described (Hogan 1994). Southern blotting with a human GDNF cDNA probe identified the founders and the offspring of the transgene-positive mice by PCR from tail DNA. Primers for human GDNF were from Exon 1 (5'-TGT CGT GGC TGT CTG CCT GGT GC-3') and Exon 2 (5'-AAG GCG ATG GGT CTG CAA CAT GCC-3'). Five different founder lines with different transgene copy numbers were used in the study.

Mouse NRTN cDNA (Genebank accession number U78109) was cloned into the *Xba*I site of the pEF-BOS vector under the human EF-1α promoter in the sense orientation (Mizushima and Nagata 1990). The correctness of the construct was verified by sequencing. The 2.5 kb fragment containing the EF-1α promoter, NRTN cDNA and polyadenylation sequences was released from the construct with *Hin*dIII and *Pvu*I and injected into the pronuclei of newly fertilised mouse eggs to produce transgenic mice as described above. Transgene positive mice were identified by PCR from tail DNA using primers TTCTCCTTGGAATTTGCCCTT and AAAGTTCTCGAAGCTCCACCG (product 338 bp). The copy number of the integrated transgene in different transgenic lines was determined by Southern blotting hybridisation with mouse NRTN cDNA probe. Five lines from different individual founders with various copy numbers of the transgene were used in the study. The FVB inbred strain mice were used for generating transgenic mice.

3.1.2 GDNF+/- mice

The GDNF deficient mouse strain was previously generated by homologous recombination to replace the exon-3-derived coding sequences (amino acids 52 to 1991) (Pichel et al. 1996). The wild-type GDNF allele was detected by PCR using the primers 5' –CCA GAG AAT TCC AGA GGG AAA GGT C and 5' -CAG ATA CAT CCA CAC CGT TTA GCG G. The targeted GDNF allele was identified by PCR utilising the primers 5' –GAT CCC CTC AGA ACT CGT and 5' –CTG TGC TCG ACG TTG TCA CTG. The homozygous mice (GDNF-/-) die within 24 hours after birth due to the lack of kidneys and intestinal innervation (Pichel et al. 1996). Spermatogenesis was analysed using heterozygous (GDNF+/-) mice and wild type littermates.

3.2 Germ cell live morphology

Seminiferous tubules were dissected and squashed on preparation slides with PBS buffer. Microscopy analysis of live cells was carried out as described (Parvinen et al. 1997).

3.3 Histology, immunohistochemistry, cell proliferation and apoptosis

For histology, freshly dissected testes and epididymides were fixed in Bouin's solution or 4% paraformaldehyde for 2-24 hours depending on the size of the biopsy, processed in paraffin, cut at 5-7µm and stained with hematoxylin/eosin. Rat monoclonal antibodies to EE2 (1:200) (Koshimizu et al. 1995, provided by Dr. Y. Nishimune), Tra98 (1:1000) (Tanaka et al. 1997, provided by Dr. Y. Nishimune), and GATA1 (1:50) (Santa Cruz CA.) were incubated on the deparaffinised sections overnight followed by PBS washes, anti-rat IgG secondary antibody at 1:500, and the standard biotin-streptavidin-peroxidase labelling procedure according to the manufacturer's instructions (Vector Laboratories, Inc. CA.). Species-crossreactive rabbit polyclonal antibodies to GDNF (1:500; Santa Cruz, CA.), c-Kit (1:200; Santa Cruz, CA.), and c-Ret (1:500; IBL, Fujioka, Japan) or a polyclonal Ret antibody provided by Dr. M. Santoro (1:100; Viglietto et al. 2000) were incubated for 48 hours at + 4 ^{o}C, after PBS washes followed by anti-rabbit IgG secondary antibody at 1:500 incubation and biotin-streptavidin-peroxidase labelling detection as mentioned above. To assay cell proliferation, the mice were injected intraperitoneally with bromodeoxyuridine (BrdU) and 5-fluoro2'-deoxyuridine (FrdU) cocktail (Amersham) at a concentration of 1-2ml/100g body weight in PBS. After 2 hr, the mice were sacrificed by neck dislocation and autopsied; testes were removed and fixed in Bouin's solution. Testes were processed in paraffin; 7µm sections were cut and deparaffinised. BrdU incorporation was detected by indirect immunofluorescence with mouse monoclonal antibodies to BrdU (Amersham) followed by rhodamine-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, Inc.). Apoptotic cells were detected in deparaffinised sections with ApopTag *in situ* cell death detection kit (Intergen) according to the manufacturer's instructions.

3.4 Total RNA isolation and Northern blotting

Total RNAs were isolated with the TRIZOL Reagent kit (Life Technology) according to the manufacturer's protocol and 30µg of total RNA was used in each lane. Northern blotting was carried out according to standard protocol (Sambrook et al. 1989) The probes (listed in Table 2) of mouse NRTN, rat GFRα2, mouse GDNF, mouse full-length c-Ret 3.3 kb, and mouse GFRα1 cDNA labelled with [α^{32}p]dCTP were used in the hybridisation. The filters were exposed overnight to a FujiBAS phosphoimager screen and the screen was scanned and analysed in the FujiBAS-1500 phosphoimager (Photo Film Co. Lt, Japan).

3.5 *In situ* hybridisation

Radioactive *In situ* hybridisation was performed as described (Wilkinson and Green 1990). Antisense and sense cRNA probes (listed in Table 2) were synthesised using

appropriate RNA polymerases and ^{35}S-labelled UTP. Hybridisation temperature was 52^{o}C and autoradiography slides were exposed at +4^{o}C for 2-4 weeks. The counter stain for the histological structure was made with hematoxylin staining. The slides were photographed with an Olympus Provis microscope equipped with a CCR camera (Photometrics Ltd). In PhotoShop 4.0 program, the dark field images were inverted, artificially stained red and combined with the bright field images.

Nonradioactive *in situ* hybridisation was performed by using the digoxigenin-UTP labelled Ret and GFRα1 cRNA probe (see Table 2), paraffin testis sections were used, and the hybridisation temperature was 70^{o}C. After hybridisation washes, the alkali phosphotase conjugated anti-digoxigenin antibody was used for the signal detection, followed by a colorimetric detection with reagents NBT and BCLP (X-Phosphate).

Table 2. Probes used in in situ hybridisation and Northern hybridisation

Probe	Size	Nucleotides	Accession number	Used in
human GDNF	636bp	full coding region	L15306	I-III
mouse GDNF	328bp	exon 3	U36449	I, II
mouse Ret	3348bp	1-3348	X67812	I
mouse Ret	684bp	2534-3217	X67812	I- IV
mouse GFRα1	777bp	1-777	AF012811	I, III, IV
rat GFRα2	2016bp	full length cDNA	AF003825	IV
mouse NRTN	1000bp	full length cDNA	U78109	IV
mouse LFng	866bp	1-866	NM 008494	III
mouse Notch 1	573bp	3901-4474	NM 008714	III
mouse Notch 2	570bp	4546-5116	NM 008715	III
mouse 3ß-HSD	360bp	1323-1686	AY046512	III
mouse WT1	1400bp	full coding region	M55512	III

3.6 Immunoprecipitation and Western blotting for GDNF and NRTN proteins

Freshly dissected testes were homogenised and lysed in high salt lysis buffer (300 mg protein/ml; high salt lysis buffer: 1M NaCl, 100 mM Tris-HCl, pH 8. 2 % BSA, 4 mM EDTA, 0.2 % Triton X-100, 2 mM PMSF, 1 mM sodium orthovanadate and 1 protease inhibitor cocktail tablet/10 ml (Complete, Mini EDTA, Boehringer Mannheim)). The lysates were immunoprecipitated with a rabbit polyclonal antibody to human recombinant GDNF peptide that crossreacts with mouse GDNF (R&D Systems) and Protein A-Sepharose. The immunoprecipitates were run on a 20 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Hybond ECL nitrocellulose membranes (Amersham Life Science). The membranes were blocked with 5 % BSA and immunoprecipitated GDNF was detected with a species-crossreactive rabbit polyclonal antibody to GDNF (Santa Cruz Biotechnology) at room temperature for 2 hours. Detection was accomplished by using anti-rabbit secondary antibody conjugated to horseradish peroxidase (Sigma) and ECL chemiluminescence (Amersham) according to the manufacturer's instructions.

Testes were homogenised in the same high salt buffer as described above. The proteins were separated on 20% SDS-polyacrylamide gel, blotted on Hybond ECL membrane (Amersham), and probed with the rabbit polyclonal anti-NRTN antibody (Chemicon) at 1:2000 dilution. Signals were detected by an ECL plus kit (Amersham, RPN 2132).

3.7 DNA and karyotype analysis

Flow cytometric analysis of the DNA contents of testicular tumour cells was performed by FACScan flow cytometry (Becton Dickinson) after ethidium bromide labelling of the nuclei (CellFIT Cell-Cycle Analysis version 2.01.2). Mouse spleen cells served as diploid cell controls. Karyotyping was done either from frozen tissues or 60 µm sections of paraffin embedded material.

3.8 Immunoprecipitation and Western blotting of phosphorylated Ret

Homogenisation of samples (either freshly dissected or frozen tissues) was performed in lysis buffer (50mM HEPES, 1% Triton X-100, 50mM sodium chloride, 50mM sodium fluoride, 10mM sodium pyrophosphate, 1% aprotinin, 1mM phenylmethylsulfonylfluoride, 0.5mM sodium vanadate). After 30 min incubation on ice the homogenate was centrifuged at 13,000g for 2 minutes, and the supernatant was analysed by either immunoprecipitation-Western blot or Western blotting. The lysates were immunoprecipitated with rabbit polyclonal antibody to human Ret cross-reacting with mouse Ret (Viglietto et al. 2000) (the Ret antibody was a gift from Dr. M. Santoro, University of Naples, Italy), run on a 7.5% SDS-PAGE, and blotted on Hybond ECL membrane (Amersham). Ret protein was detected by the Ret antibody and the phosphorylation of Ret was detected by monoclonal phosphotyrosine antibody (Transduction Laboratories), and the signals were detected by an ECL plus kit (Amersham, RPN 2132).

3.9 MAPK and AKT phosphorylation assays

Total protein lysates mentioned above were separated on a 12% SDS-PAGE, blotted, and probed with polyclonal antibodies to phosphorylated forms of either ERK1/2 (Promega) or AKT (protein kinase B) (New England Biolabs). The blots were then reprobed with the antibodies, which recognise both the phosphorylated and unphosphorylated forms of ERK1/2 (Promega) or AKT (New England Biolabs). The optical density of the bands was determined using a phosphoimager and a TINA program.

3.10 Germ cell isolation and transplantation

3.10.1 Depletion of host testis by gamma irradiation

NMRI$^{nu/nu}$ nude mice were anaesthetized and testes were irradiated using a Philips Orthovolt (0.5 mm Cu filter). A 2 cm diameter tube was used to confine radiation to the testes and their immediate surroundings. Fractionated doses of 1+4, 1½+8, 1½+12 and

1½+16 Gy were given with an interval of 24 h. For evaluation of the extent of depletion, testes were harvested after 3 months, fixed in Bouin's solution and embedded in paraffin.

In a minimum of 200 tubule cross sections the presence of germ cells was assessed and tubules were scored as containing endogenous spermatogenesis when spermatogonia or in addition later stages were present. Testes that had been withdrawn into the abdominal cavity out of the irradiation field during irradiation were not included in the analysis. For transplantation, mice were used 1 month after irradiation and testes were harvested 2 months after transplantation.

3.10.2 Preparation of donor cells

Germ cells were isolated from GDNF-overexpressing or wild type mouse testes, of which the correct genotype was confirmed by PCR of tail DNA. Briefly, testes were decapsulated and after tearing apart the tubule fragments, subjected to two successive enzymatic treatments of 1 mg/ml trypsin, hyaluronidase and collagenase and 1 mg/ml hyaluronidase and collagenase, respectively, in MEM containing 0.12% sodium bicarbonate, 4 mM L-glutamine, single strength nonessential amino acids, 100 IU-100 µg/ml penicillin-streptomycin, 40 µg/ml gentamycin and 15 mM HEPES. Cells were separated from remaining tubule fragments by centrifugation at 30 g and, after filtration through 77 and 55 µm nylon filters, pelleted and loaded onto a Percoll discontinuous density gradient. The purity of the cell suspensions was assessed by Nomarski optics, cells with a high nucleus-to-cytoplasm ratio and one or more distinct nucleoli being characterised as spermatogonia. Fractions with a purity of at least 40% were washed, counted and resuspended to a concentration of cells equivalent to 10^6 germ cells/ml. Aliquots of 20 µl of the germ cell suspensions were kept on ice until transplantation.

3.10.3 Transplantation

Germ cell suspensions (20 µl) were transplanted via efferent duct microinjection (Ogawa 1997) into recipient rete testes and seminiferous tubules, one month after local fractionated irradiation. Testes were analysed after two months, a time period previously shown to allow for full reinitiation of spermatogenesis (Brinster and Zimmerman 1994). Host mice were sacrificed by CO_2 asphyxia and testes were fixed in Bouin's solution.

3.10.4 Statistical analysis

Data are presented as mean±SEM. Statistical analysis was performed by nonparametric ANOVA. $P<0.05$ (two–tailed) was assumed to indicate statistical significance.

4. RESULTS

4.1 mRNA expression of *GDNF* family ligands and receptors in normal mouse testes

By Northern blotting and *in situ* hybridisation, we found that, among the GDNF family ligands, *GDNF* and *NRTN* were expressed in normal testis (I, IV). *ARTN* and *PSPN* were not detectable by *in situ* hybridisation (unpublished data), although the coreceptor *GFRα4* for *PSPN* is expressed prominently in the pachytene spermatocytes in testis (unpublished data, Lindahl et al. 2000). We therefore focused our study on the expression of *GDNF*, *NRTN*, and the expression of their ligand-binding receptors *GFRα1*, *GFRα2*, and *Ret* tyrosine kinase. Northern analysis showed a strong band for *GDNF* and its receptors *GFRα1* and *Ret* in the neonatal testis and a low density band after the second postnatal week (I), whereas *NRTN* and *GFRα2* were highly expressed after puberty (IV). *In situ* hybridisation showed that both *GDNF* and *NRTN* were expressed by Sertoli cells, while their receptors were expressed by germ cells (I, IV). The GDNF receptors *GFRα1* and *Ret* were expressed in a small subset of spermatogonial cells which might include putative spermatogonial stem cells (I), these cell are flattened on the basement membrane, mostly singly or paired shown by nonradioactive *in situ* hybridisation (Fig. 10).

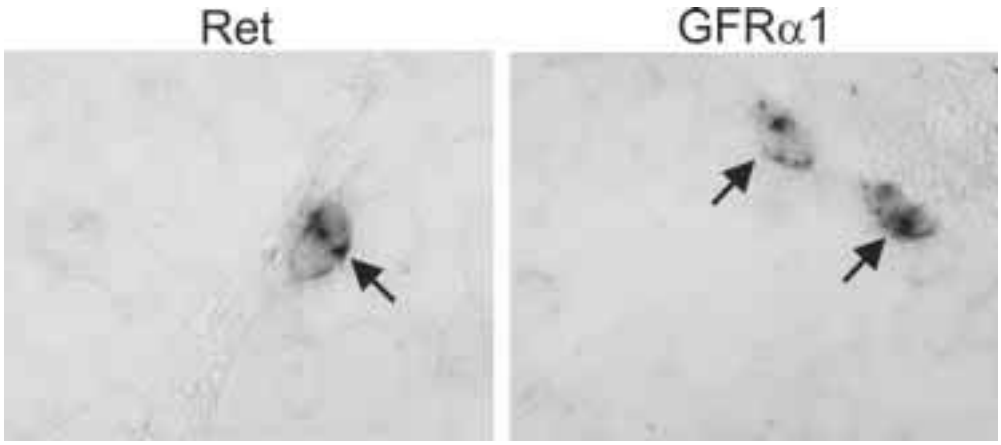

Figure 10. The mRNA expression of *Ret* and *GFRα1* in the normal adult testis detected by nonradioactive *in situ* hybridisation. Arrows point to the positive cells.

4.2 mRNA and protein expressions of GDNF and its receptors in pEFBOS-hGDNF transgenic mice

The expression of GDNF protein of pEFBOS-hGDNF construct was detectable in immunoprecipitation-Western blotting from the supernatant of the culture media of the transfected Cos cells. We generated several transgenic mouse founder lines with various copy numbers of transgene (I). The expression profile of the transgenic *GDNF* was screened by Northern blot hybridisation and the expression was restricted to the testis (I.

Web Figure 2). A ~1.0 kb transgene transcript was constantly expressed from the neonatal stage throughout adulthood, but the expression was low in the first two postnatal weeks (I). The elevated protein levels of GDNF were verified from the transgenic mouse testis by Western blotting (I). In the transgenic testis, the mRNA levels for GDNF receptors *GFRα1* and *Ret* were constantly upregulated (I, III). By Western blotting, the increased Ret protein and phosphorylation levels were detected in the arrested spermatogonial cells (data not shown) and in tumour cells in the GDNF-overexpressing transgenic mice (III). Immunolocalisation of Ret showed a wider expression than Ret mRNA expression. Ret proteins are localised in spermatogonia and earlier spermatocytes in wildtype littermates (II), and in the arrested spermatogonial and tumour cells in transgenic mice (I, IV).

4.3 Testicular phenotype in GDNF+/- mice

The GDNF-deficient mice generated by Pichel et al. (1996) die in the first postnatal day. Haploinsufficiency of the GDNF gene for kidney development with unilateral renal agenesis or rare dysagenesis has been observed in the heterozygous (GDNF+/-) mouse (Moore et al. 1996; Pichel et al. 1996; Sánchez et al. 1996). The GDNF+/- mice were fertile, though the histological analysis of testis revealed approximately 50% atrophic seminiferous tubules with germ cells depletion or Sertoli cell-only (I). In testes of 7-9 weeks old mice, spermatids or spermatozoa were present in most atrophic seminiferous segments, but the spermatogenic cells including spermatogonial cells were rarely present, if not completely absent. The BrdU labelling showed very few mitotic spermatogonial cells in the basal compartment of the atrophic segments that had not yet progressed to Sertoli cell-only (I). At old age, atrophic tubules become Sertoli cell-only.

4.4 Testicular phenotype in pEFBOS-hGDNF transgenic mice

The transgenic mice with testis-restricted overexpression of GDNF were healthy, and no abnormalities were found in any tissues, except for the testis. Testicular morphology of these mice was normal in neonates. After 2 to 3 weeks of age, a chimeric histological pattern was observed. Tubular cross sections not only showed normal spermatogenesis but also displayed large cell clusters. The cells in the clusters were first characterised as type A spermatogonia by morphological analysis of live cell (I. Web Figure 3) and by histology showing only little heterochromatin (I). The cluster cells expressed spermatogonial markers EE2 (I) and Tra98 (data not shown). Further clonal characterisation showed that spermatogonial cells in the clusters were likely undifferentiated spermatogonia, because of the absence of the synchronised mitotic or apoptotic figures of these cells; the cluster cells were mostly singly or in small clones in pairs, groups of four, and eight. C-kit is an antigen of differentiating spermatogonia (Schrans-Stassen et al. 1999). The cluster cells were negative for c-kit (I. Web Figure 4).

The cell proliferation was analysed by bromodeoxyuridine (BrdU) incorporation. After the first postnatal week in mice, the normal spermatogonial proliferation displays a segmental pattern in tissue cross-sections. In transgenic mouse testes this stage-specific cell proliferation was disrupted, the cells within clusters from every segment were

labelled (I). The average proliferation indexes of spermatogonia in 100 transsections of wildtype and transgenic tubules were not significantly different, and the peak proliferation index was higher in wild type than in transgenic mice (I). TUNEL label for apoptosis cells showed that the transgenic mice had more apoptotic germ cells after 2-3 weeks (I). Moreover, in response to a differentiating signal (retinoic acid), spermatogenesis was partially restored in transgenic mice and the cells in clusters were rapidly induced to undergo massive apoptosis (I).

The somatic cells of seminiferous tubules were normal as compared to the wild type testes in several respects. Sertoli cells showed no significant alteration in their number, and expressed GATA1 (I. Web Figure 4). The Sertoli-Sertoli cell tight junctions were formed in transgenic mice (I). The interstitial cells displayed hyperplasia. However, serum T and FSH levels were not different between transgenic and wild type mice (unpublished data). Non-metastatic testicular tumours were occasionally developing at the age of 7 months and regularly in old GDNF-overexpressing transgenic mice after the age of one year.

4.5 Transplantation of GDNF-expressing germ cells

Analysis of endogenous repopulation of germ cell in irradiated testes was performed 3 months after irradiation (II). In the irradiated mice, a radiation dose-dependent decrease in the amount of tubule cross sections containing spermatogenic cells was found. For the immunodeficient NMRI nude mice, the strain intended as recipient for transplanted germ cells, the dose of 1½ +12 Gy resulted in a depletion of spermatogenic cells in 97±1% seminiferous tubules (n=6).

Transplantation of germ cell suspensions isolated from GDNF overexpressors resulted in the formation of spermatogonial clusters in all recipient testes. Clusters were of variable size and could be recognised by the accumulation of A spermatogonia-like cells with a high nucleus-cytoplasmic ratio and a relative paucity of heterochromatin. Clusters and more advanced stages of spermatogenesis were found in the same tubule cross sections (II). These more advanced germ cells did not proceed beyond pachytene spermatocytes (II). In addition to cluster-containing tubules, tubules that contained only a rim of spermatogonial cells were found (II). The basal stretch and clustered spermatogonia formed by the transplanted transgenic germ cells expressed Ret protein (II) and transgenic *GDNF* mRNA (data not shown).

In the testes of the two mice transplanted with wild type cells, neither clusters nor rims of spermatogonial cells were noted (data not shown). Here, normal spermatogenesis was found in 24.5% and 20.5% of the seminiferous tubules, respectively, and germ cells in these tubules usually had advanced to the pachytene spermatocyte stage, in line with previous transplantation studies.

4.6 Characterisation of testicular tumours in old pEFBOS-hGDNF transgenic mice

Old transgenic mice with overexpression of GDNF in undifferentiated spermatogonia developed malignant testicular tumours. The characterisation of the tumour type is

presented in (III). 56% of the tumours were bilateral. Most tumours appeared at around 1 year of age, though the earliest case was identified in a 7 month-old mouse.

All tumours were histologically uniform. They were composed of round-shaped cells with scant cytoplasm, and fibrous bands between tumour nodules were commonly present, but no large clusters of lymphocytes were observed. The mitotic index of the tumours was constantly high (~10 mitotic figures/40 x magnification), and the mitotic figures were often atypical. No distant metastases were found by autopsy and histological analysis.

Tumour cells expressed germ cell markers EE2 and Tra98, but were negative for Leydig cell and Sertoli cell markers (*3β-HSD* and *WT1*). In the histochemical staining for alkaline phosphotase, the tumour cells showed a strong enzymatic reactivity. In addition, the enzyme activity was also seen in a few cells in the spermatogonial clusters of young transgenic mice. Other molecules characterising both tumour cells and spermatogonial cells in the clusters of young transgenic mice were Notch signalling molecules, *i.e*. *Notch1*, *Notch2* and lunatic fringe (*L-Fng*).

Tumour cells expressed the transgenic *GDNF* , *GFRα1* and *Ret* as detected by *in situ* hybridisation. Elevated levels of hyperphosphorylated Ret protein were shown by immunoprecipitation-Western blotting. We also found increased levels of phosphorylated AKT (protein kinase B) and MAPK (Erk1/Erk2) in the tumour cells.

The DNA contents of tumour cells as analysed by flow cytometry exhibited an aneuploid karyotype. A distinct triploid peak was always observed, whereas a considerable number of tumour cells appeared to be hypodiploid. No peak at the tetraploid or higher levels was found. The atrophic tumour-free testes from the transgenic mice (at the age around 3-10 months) showed diploid DNA content in flow cytometric analysis although the tripolar cells were occasionally observed.

4.7 Testis deficiency in pEFBOS-NRTN transgenic mice

In the transgenic mice overexpressing NRTN in testis, only a partial penetrant and transient testicular phenotype was observed (IV). Spermatogenic defects were observed in each of the five transgenic lines at the age of 3-5 weeks. In some transgenic mice, round spermatids and pachytene spermatocytes degenerated in some seminiferous tubules, while simultaneously in others spermatogenesis preceded normally. In other transgenic mice, the rete testis was dilated, and the layers of spermatids and late spermatocytes were reduced in all seminiferous tubules. After 5 weeks of age, no abnormalities were found in spermatogenesis anymore and the NRTN overexpression mice were fertile, even though the transgene expression continued during adulthood, as evidenced by Northern and Western blotting. No testis tumours were observed in these transgenic mice.

5. DISCUSSION

5.1 Paracrine regulation of germ cells by GDNF and NRTN

Sperm differentiation can be regulated by local paracrine signals between germ cells and Sertoli cells. Sertoli cells, the most important regulators of spermatogenesis, secrete a signal molecule, GDNF, acting on the target cells, probably the undifferentiated spermatogonia that possess Ret and GFRα1, the receptors for GDNF. Therefore, the reciprocal interplay of GDNF from Sertoli cells and its receptors on spermatogonial cells fulfils the criteria for a paracrine signalling system for regulating spermatogonial differentiation. According to the sites of mRNA expression for the receptors for GDNF, the target cells for GDNF are a small subgroup of undifferentiated spermatogonia including the putative spermatogonial stem cells (I, Fig. 10). However, *in situ* hybridisation is insensitive when a certain mRNA has low levels of expression. Whether there is low expression of *Ret* mRNA in other cell types is not clear. In fact, Ret proteins were detected by immunohistochemistry also in early spermatocytes in addition to the basal spermatogonial population (II). A low level of transcription coupled with a highly efficient rate of translation might explain this. It may also be due to Ret proteins being allocated to the daughter cells during cell divisions, or being directly transferred between the germ cells through the intercellular bridges. Therefore it cannot be excluded that GDNF might affect a larger range of spermatogonial cell types.

Sertoli cells also produce another member of the GDNF family, NRTN, after puberty. Its high affinity coreceptor *GFRα2* is expressed at the same time in spermatids and weakly in spermatocytes. A possible paracrine interaction between Sertoli cells and germ cells may take place via the interplay of NRTN and GFRα2. However, Ret expression was not detected in the *GFRα2* expressing cells by *in situ* hybridisation. It might be that a low expression of Ret is not detectable in these cells. It is also possible that NRTN signals through a Ret-independent signalling pathway. The biology and function of a Ret-independent GFRα1 signal pathway have been demonstrated recently (Poteryaev et al. 1999; Trupp et al. 1999), but possible Ret-independent signalling via GFRα2 remains to be identified. The transcripts of *GFRα2* are smaller in the testis (1-2 kb, IV) than in the somatic tissue (4kb, Baloh et al. 1997). The identity of GFRα2 transcripts in testis is unknown. However, one is big enough to code for the mature protein when compared to the length of the coding area of the gene (Baloh et al. 1997). Whether this transcript encodes for a functional protein needs to be studied in the future.

5.2 GDNF regulates spermatogonial self-renewal and differentiation

Normal stem cell renewal and differentiation should be in the balance. Excess self-renewal over differentiation would reduce the seminiferous epithelium and enhance stem cell formation. If differentiation prevails, the stem cells would deplete themselves, leading to seminiferous tubules with only Sertoli cells (reviewed by de Rooij 2001).

Many factors are proposed to regulate spermatogonial cell divisions. The focus has been on the molecules involved in the differentiation of Aal into A1 spermatogonia (reviewed

by de Rooij and Russell 2000; de Rooij 2001). However, the molecular control of self-renewal and differentiation of stem cells has remained enigmatic. Now we have shown by loss-of-function and overexpression transgenic models that the dosage of GDNF may regulate the cell fate decision of undifferentiated spermatogonia. Low dosage GDNF favours the differentiation of spermatogonia, high dosage favours the self-renewal. The haploinsufficiency of GDNF in GDNF+/- mice led to a depletion of tubular germ cells including stem cells, leading to approximately 50 percent of the tubules with Sertoli cells-only. However, in the GDNF+/- mouse testis, the first wave of spermatogenesis proceeded in the degenerating tubules, as evidenced by the presence of spermatids and spermatozoa. We assume that the stem cell self-renewal is reduced due to the decreased GDNF dosage, with differentiation exceeding self-renewal leading to the depletion of seminiferous epithelia. In the opposite situation, when the GDNF dosage is increased, differentiation of spermatogonia is inhibited. Tubules in 3-week-old GDNF-overexpressing mice contain large clusters of undifferentiated spermatogonia. The transplantation of these clustered cells to a wild type host mouse suggested that the arrest of spermatogonial differentiation is donor cell-intrinsic and is caused by deregulated autocrine GDNF signalling.

GDNF is a small molecule, which normally is secreted by Sertoli cells. As no Sertoli cell-specific promoter is available at the moment, GDNF could not be targeted to the cells that normally express it. Therefore, one should note that the GDNF overexpression mouse does not recapitulate the normal paracrine action of GDNF, and instead, an autocrine signalling loop is formed in the spermatogonia that express both the transgenic GDNF and the receptors for GDNF. Unlike SCF, in addition to the soluble form secreted from Sertoli cells, the Sertoli cell membrane-bound isoform of SCF is also crucial for the regulation of germ cell development (Mauditt et al. 1999). No evidence has been presented for the existence of a membrane-bound isoform of GDNF protein. We thus assume that the germ cell targeted expression of GDNF may mimic the GDNF protein secreted from Sertoli cells. The cells which express the receptors Ret and GFRα1 are evidently the GDNF targets. A small subset of spermatogonia, very likely including putative the stem cells, are the primary target for GDNF, because they express both Ret and GFRα1. The morphology and nature of small clonal size for the cells within clusters (I: no intercellular bridges, most singly, a few in pairs or small chains) suggest that the clustered cells arrested by GDNF are undifferentiated cells including the putative stem cells. Moreover, the capability of these cells to colonise and repopulate the host seminiferous tubules after transplantation suggests that they have stem cell properties. Thus the spermatogonial stem cell is at least one, if not the only, target for GDNF signalling.

5.3 NRTN in spermatogenesis

NRTN and GDNF overexpressing mice show different phenotypes. Unlike the GDNF-overexpressing mice, the NRTN mice show no accumulation of spermatogonia, they have only a low penetration of spermatogenic defects, and when present, these are transient and recover after the first spermatogenic wave, and therefore the mice are fertile. In contrast to the function of GDNF as a regulator of spermatogonial differentiation, NRTN

may affect the development and survival of spermatocytes and spermatids. The phenotype of the dilated rete suggests that NRTN might also regulate fluid production and reabsorption. Therefore, proper expression of NRTN might be important for normal spermatogenesis.

The distinct functions of GDNF and NRTN might result from the non-overlapping distribution of the ligand-binding GFRα1 and GFRα2 receptors. Because the testis phenotypes of GDNF and NRTN overexpressing- and deficient-mice are completely different, biologically significant cross talk between NRTN and GRFα1, and on the other hand between GDNF and GFRα2, is unlikely. GDNF and NRTN employ the same signalling receptor Ret. However the *Ret* receptor is only colocalised with the cognate ligand-binding coreceptor *GFRα1*, but not *GFRα2*. Since we have not seen Ret mRNA and protein in the spermatids and late spermatocytes, either the *Ret* levels are very low in these cells or we are dealing here with Ret-independent, GFRα2 signal transduction. One more alternative, NRTN may also act upon an unknown transmembrane receptor to regulate germ cell development and fluid metabolism.

5.4 Promotion of seminomatous tumours by targeted overexpression of GDNF

5.4.1 Cell proliferation, differentiation and apoptosis

Since large numbers of spematogonial cells are formed in GDNF-overexpressing mice during the third postnatal week, it is logical to assume that GDNF may promote cell proliferation, and prevent these cells from differentiation and apoptosis. Therefore, these cells accumulate and form clusters. However, in normal spermatogenesis the cell density is strictly controlled by an unknown regulatory mechanism(s), which triggers apoptosis. It has been suggested that Sertoli cells actively regulate the optimal number of spermatogenic cells (reviewed by de Rooij and Loc 1987; de Rooij 2001). Apoptosis was started to increase during the two-third weeks, but the peak apoptosis appears at four weeks of age in these transgenic mice. The delay for eliminating the cells in the clusters suggests that GDNF may prevent the cells from entering apoptosis. Why are the clustered cells eventually eliminated from the luminal compartment? The occurrence of high levels of apoptosis correlated to the formation of the blood-testis barrier. After the formation of the blood-testis barrier, spermatogonia are normally located in the basal compartment outside the barrier. Thus, the reason for the disappearance of the clusters might be the misallocation of cells where the nutritional and biochemical environment is not optimal for supporting their survival. The blood-testis barrier may hinder the transgenic spermatogonial cells from forming new clusters intruding into the lumen. Instead, these spermatogonial cells preferentially spread in the basal compartment at adulthood. This rim of spermatogonia is unable to differentiate due to the constant overexpression of GDNF, but they do not die either. The prevention of apoptosis and differentiation of these cells may raise the risk of tumour transformation. Indeed, the mice developed testicular tumours regularly at the age of one year.

5.4.2 Hypothetical mechanism(s) for the development of testicular tumour

The regular development of seminomatous germ cell tumours suggests a common mechanism for this type of tumour formation. In addition to the oncogenesis of MEN 2-Ret, oncogenic mutations of many receptor tyrosine kinases are well known. However, protein-tyrosine kinases (PTKs) and the downstream kinases are important regulators of intracellular signal-transduction pathways. Perturbation of PTK signalling by mutations and other genetic alterations results in deregulated kinase activity and malignant transformation. The lipid kinase PI3-K and some of its downstream targets, such as the protein-serine/threonine kinases AKT and p70 S6 kinase, are crucial effectors in oncogenic PTK signalling. Oncogenic conversion of protein kinases results from perturbation of the normal autoinhibitory constraints on kinase activity (reviewed by Blume-Jensen and Hunter 2001). The MEN 2 cancer syndromes are critically dependent on the activation the PI3 kinase/AKT (Segouffin-Cariou and Billaud 2000; De Vita et al. 2000; Murakami et al. 1999). The overexpression of GDNF induced up-regulation and hyperactivation of Ret receptor tyrosine kinase (III). Ret tyrosine kinase and its downstream effectors including MAP kinases (Erk1/2) and AKT are constantly activated in tumour cells. Thus the mitogenic effects of MAP kinase and the antiapoptotic effect of AKT may be initial risk factors for tumour formation. AKT promotes cell survival by its inhibitory effect on cell apoptosis. Gene inactivation of *akt1* has led to the increase of germ cell apoptosis, especially spermatogonial apoptosis (Chen et al. 2001). Therefore, the hyperactivity of AKT in the spermatogonial cells in GDNF-overexpressing transgenic mice may play a role in the oncogenic transformation of these cells. MEN 2 transgenic mice had a variable latency period for the development of medullary carcinoma. This finding supported the view that additional genetic alterations are required for malignant transformation of thyroid C cells (reviewed by Takahashi 2001). Ret cooperates with RB/p53 inactivation in a somatic multiple-step model for murine thyroid cancer (Coxon et al. 1998). This finding suggests that the development of a murine neuroendocrine tumour requires mutational dysregulation within both receptor tyrosine kinase and nuclear tumour suppressor gene pathways. The second hit(s) of the malignant transformation in GDNF overexpressing transgenic mouse testis remains to be identified. Mutation or a deregulation of tumour suppressor genes may be the second step required for the oncogenic transformation. Retinoblastoma family proteins encompassing pocket proteins pRb, p107 and p130, are potentially important regulators of cell growth, differentiation and apoptosis. The retinoblastoma susceptibility gene, Rb, was the first tumour suppressor gene described. By regulating gene expression, pocket proteins control cell cycle progression, cell cycle entry and exit, cell differentiation and apoptosis (Graña et al. 1998). Roles of pocket proteins in the control of germ cell proliferation, differentiation and apoptosis have been suggested (Yan et al. 2001). They are expressed in several cell types in the testis, which include spermatogonia. Interestingly, p130 is localised in the undifferentiated spermatogonia (Yan et al. 2001). Whether there is a deregulated activation of these proteins during testicular tumour formation will be addressed in our future studies.

5.4.3 GDNF-induced testicular tumours resembling human seminoma

Several characteristics of the GDNF-induced tumours resemble the classic seminoma rather than spermatocytic seminomas. Firstly, the histological features, secondly, the triploid karyotype of tumour cells, thirdly, the primordial feature of tumour cells which is characterised by the high activity of alkaline phosphotase, fourthly, the possibly origin of tumour cells from "CIS" cells. In the transgenic mice, immature gonocyte-like germ cells, which seem to be comparable to CIS cells in man, are present before tumours appear. Human CIS cells are PLAP positive and are unable to undergo differentiation, slowly replacing the seminiferous epithelium (Skakkebaek and Berthelsen 1978, 1981; Skakkebaek et al. 1987, 1998, 2000). We found clusters of undifferentiated spermatogonia already at two weeks of age and the first microinvasive spermatogonia already at seven months of age, further indicating that the pathogenesis of these tumours can be traced back to a young age. Tripolar cell divisions and the alkaline phosphotase positive cells in the clusters of the young GDNF overexpressing mice may suggest that these cells are already transformed, resembling the CIS cells.

Although these experimental murine tumours and human seminomas are similar in several respects, there are also differences, such as the high frequency of bilateral tumours in mouse (unilateral in man), the appearance of tumours at an old age in mouse vs. at young age in man, tumour development in the context of severely distorted spermatogenesis in mouse vs. normal spermatogenesis in man, and the absence of large lymphocyte infiltrates in mouse while these are present in most but not all classic seminomas (Mostofi 1980; Skakkebaek et al. 1987). C-kit is negative in mouse vs positive in human seminoma. These differences may suggest species variation in the disease.

In addition to the hypothesis that GCTs originate from CISs, it was also proposed that perhaps malignant transformation of type A spermatogonia is associated with dedifferentiation of adult germ cells so that they revert structurally and functionally to more primitive precursor cells (Holstein et al. 1987). This hypothesis has not received direct evidence. However, since the majority of the cells are diploid prior to the development of testicular cancer, it cannot be absolutely excluded that the tumour cells are derived from transformed A spermatogonia by dedifferentiation, although the possibility of CIS cell origin is evidenced by the several aspects as discussed above. According to another speculation, the target cell type for transformation in germ cells may be zygotene-pachytene spermatocytes with replicated chromosomes that express wild-type p53, harbour DNA breaks, and may be prone to apoptosis (Chaganti and Houldsworth 2000). Our data does not support this hypothesis, because the DNA measurement of GDNF-overexpressing transgenic mice show a diploid karyotype and no tetraploid DNA content, and histological analysis reveal that no zygotene or pachytene spermatocytes were present at the stage prior to the development of testicular tumours. Therefore it is unlikely that the tumour cells are transformed from zygotene or pachytene cells. Although no triploid content has been observed in flow cytometry analysis, the tripolar spermatogonial cells in young as well as old transgenic mice probably represent aneuploid cancer precursor cells with triploidy. The number of these cells might be too few to be shown by flow cytometry.

6. CONCLUDING REMARKS AND PERSPECTIVES

This thesis work reveals novel functions for GDNF and NRTN in the regulation of spermatogenesis. The control of cell fate decisions of undifferentiated spermatogonia by GDNF renders the molecules affecting GDNF signalling potential targets for developing male contraceptives. The GDNF-deficient and overexpressing transgenic mouse lines serve as useful tools for studying the pathogenesis of infertility. Furthermore, the testicular tumours developing in the GDNF-overexpressing mice resemble human seminomatous GCTs. This transgenic mouse line is the first animal model for seminoma and therefore serves as a powerful tool to study the pathogenesis of seminoma and to test novel therapies and diagnostic methods.

The spermatogonial stem cell transplantation techniques could be used not only for conducting basic research of germ cell biology, but also for restoring the spermatogenesis of a man who suffers from infertility or gonadal damage after chemotherapy. The testicular tumours formed in GDNF-overexpressing transgenic mice are a consequence of long term stimulation by a GDNF signal loop *in vivo*. It is unlikely that spermatogonia would undergo transformation during a short term *in vitro* stimulation. As we have discovered, stem cell self-renewal overrides differentiation in GDNF-overexpressing transgenic mice. This finding suggests that GDNF may also be useful *in vitro* for multiplying the stem cell population prior to germ cell transplantation, which might be beneficial for the patients who need to cryopreserve spermatogonic cells prior to oncogenic therapy. However, the risk of cancer should be taken into consideration when reintroducing the cryopreserved cells back into the patients. Similar precaution should be considered in the development of GDNF-based therapies for the treatment of neurodegenerative diseases.

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